IN VITRO MODELLING OF THE IMMUNOLOGICAL INTERACTIONS BETWEEN THE SALMON LOUSE, *LEPEOPHTHEIRUS SALMONIS* (KRØYER, 1837), AND THE ATLANTIC SALMON, *SALMO SALAR* (L., 1758)

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I declare that this thesis has been composed by myself and is a record of work carried out by myself, unless otherwise stated. All quotations have been distinguished by quotation marks and all sources of information acknowledged. This thesis has not been presented or accepted in any previous application for a higher degree.

Ricky Butler
April 2001

ABSTRACT

Atlantic salmon, *Salmo salar*. L., have been shown to be more susceptible to infections by the caligid copepod *Lepeophtheirus salmonis* (Krøyer, 1837) than other salmonid species. Atlantic salmon exhibit a reduced cellular immune response to the attached parasite which has led to the hypothesis of the presence of sea louse associated compounds that depressed the fish's normally efficient inflammatory mechanisms.

The aim of the current study was to test this hypothesis. A biochemically defined *in vitro* culture system was developed that would allow collection of the secreted/excreted products of the copepodid larvae of *L. salmonis*, and avoid their contamination by metabolites of the host from their collection, *in vivo*.

Available tissue culture methods proved inadequate in supporting copepodid culture because of the louse requirement for a seawater maintenance medium that was osmotically unsuitable for cultured cells. Tissue engineering technologies developed in the construction of human living skin equivalents were successful in the development of an Atlantic Salmon Skin Equivalent (ASSE). ASSE is a novel organotypic tissue culture substrate that was constructed from Atlantic salmon fibroblasts (AS-6) and primary cultures of Atlantic salmon epithelial cells. Cells were supported in a matrix of collagen fibres, acid extracted from the tails of rats, and combined using a layering technique to create a substitute salmon skin. ASSE has a fibroblastic dermal equivalent overlaid by an epidermis-like layer and a layer of collagen, and was maintainable in a seawater media. During its development, the cells within ASSE showed signs of differentiation that included stratification, increased fibronectin production by cultured fibroblasts, and the formation of a basement membrane-like layer at the junction of the dermis and epidermis. In this environment, ASSE allowed the survival of copepodid larvae for an average of 12 days, a period approximately 5 days longer than that of their free-swimming counterparts. Furthermore, cultured copepodids were observed feeding, exhibiting the normal range of settlement behaviours, and also showed increases in their length. However, metamorphosis to the chalimus I stage did not occur and was not stimulated by the supplementation of ASSE with salmon mucus, salmon peptone or DL methionine.

Nevertheless, copepodid maintenance on ASSE allowed the collection of the compounds they released into the culture media. These were assayed for their effects on the immunological functioning of salmon macrophages. These assays demonstrated that the chemotaxic ability of

macrophages was significantly reduced following treatment with louse culture supernatants (LCS), as was their phagocytic ability. In both assays, the activity of each function was approximately 50% lower than that recorded in untreated cells. Intracellular respiratory burst and the phagocytic index of active phagocytes, however, was not affected. The biological activity of LCS was inhibited following heating, dilution, and treatment with proteinase K, indicating that the active immunosuppressive compounds were either themselves proteins, or required protein to be effective. The active substance was designated Louse Immunomodulatory Factor (LIF).

The enzyme profiles of culture supernatants were investigated using API ZYM test strips. The profiles of LCS supernatants were significantly different to those of control supernatants from the early stages of louse incubation with ASSE. LCS supernatants showed elevated levels of leucine aminopeptidase, C4 and C8 esterases, alkaline phosphatase, β-glucuronidase, and N-acetyl-β-glucosaminidase. The involvement of these enzymes in the digestive processes of arthropods is well documented. However, the presence of the chitin hydrolysing enzyme N-acetyl-β-glucosaminidase, may also be suggestive of the preparation of copepodids for moulting. This, and the involvement of these enzymes in digestion in copepodids, and as possible immunomodulatory compounds is discussed.

Gel filtration chromatography identified 14 proteins in the LCS that were not present in the control supernatants. These proteins were in the molecular weight range <1 kDa to 2665 kDa. No biological activity was attributed to these proteins when isolated by chromatography and assayed for their effects on macrophage chemotaxis. This lack of activity may be associated with their dilution during the chromatography process in which the supernatants were diluted approximately 5000 times. Experimental evidence showed that the activity of LIF was absent when supernatants were diluted to 1:1000, and so methods of concentration may be required in order to establish the immunological activity of these proteins.

This study has developed an Atlantic salmon skin substitute that could have a great number of applications in the study of salmonid metabolism, cellular communication, immunology, and drug and chemical testing. Here, it supported the extended maintenance of sea lice larvae *in vitro* and allowed the collection of the products of their culture. The findings have shown that the copepodid stage of *L. salmonis* produces substances, LIF, that depress the chemotaxic and phagocytic activity of salmon macrophages, *in vitro*. These active substances may also be responsible for the depression of the inflammatory responses of sea lice infected Atlantic salmon, *in vivo*.

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were found to be most heavily infected with *L. salmonis* followed by pink salmon (*O. gorbuscha*), chum salmon (*O. keta*), coho salmon and sockeye salmon (*O. nerka*) (Nagasawa, 1987). In the northern North Pacific Ocean pink salmon were more heavily infected than chinook salmon, followed by coho salmon, chum salmon and sockeye salmon (Nagasawa *et al.*, 1991). Johnson & Albright (1992) demonstrated these differences following experimental infection and showed that Atlantic salmon suffered the greatest louse burden, followed by chinook salmon and coho salmon. Furthermore, differences in the developmental rates of the parasite were observed between the species, progression being slower on the Pacific species. The authors suggest that these may be caused by nutritional factors and/or the non-specific defence mechanisms of the host, particularly since they demonstrated a much-reduced inflammatory response in Atlantic salmon at the site of parasite attachment on the gills as well as the body surface. In coho salmon mild inflammation of the dermis was visible 1 day post infection, with the inflammatory response being well established by day 5, whilst only limited tissue responses were observed in Atlantic salmon at day 20. They suggest that it is the magnitude and speed of the inflammatory response in coho salmon that is responsible for the clearing of *L. salmonis* following an infection. Atlantic salmon have efficient cellular responses following both physical injury and pathogen invasion that are reviewed in Wolke (1975). However, it is evident that this response is absent during *L. salmonis* infections. The work of Johnson & Albright (1992) allows consideration of two possible factors responsible for the comparative susceptibility of these salmon species. Either, the non-specific defences of coho salmon are generally more effective than those of Atlantic salmon, or the presence of sea lice suppresses the otherwise effective Atlantic salmon inflammatory response.

This study aimed to investigate the proposition that the cellular defences of Atlantic salmon are modulated during sea lice infections, and that *L. salmonis*, in common with other arthropod parasites, secretes substances that have the effect of suppressing the host immune response to permit successful parasitism (Woo, 1992; Wikel *et al.*, 1994; Wikel *et al.*, 1996; Kotwal, 1997). Immunomodulating products from arthropod parasites have in the past been identified as normal metabolic compounds, or their analogues, including prostacyclin and prostaglandins, present within the host (Wikel, *et al.*, 1994; Wikel, *et al.*, 1996). Therefore, existing levels of these compounds may confuse their detection within the host. Radiolabelling of the parasite may allow recovery and identification of radioactive compounds secreted to the host, however, such experiments have proved difficult in the past when attempted with other multicellular parasites (Payares *et al.*, 1984; Payares & Simpson, 1985). This study, as an alternative method, proposed to develop a biochemically defined *in vitro* model that would permit maintenance of sea lice and the collection and detection of louse secreted compounds that may otherwise be

masked by metabolic background levels *in vivo*. These compounds would then be evaluated for their potential to modulate the functioning of cellular immunity *in vivo* by their incorporation into standard *in vitro* assays using salmon macrophages as the target cell.

LEPEOPHTHEIRUS SALMONIS (KRØYER, 1837)

Sea lice were first reported in the 1960's from salmon farms around the Norwegian coast and started to appear in large numbers at Scottish farms in the mid 1970's. Records of sea lice on wild salmonids for the last century show their presence but at low prevalence and abundance (Lewis, 1963; Wootten, *et al.*, 1982). This apparently balanced co-existence between host and parasite in the wild situation may persist over extended periods with no significant impact on the host (Wootten, *et al.*, 1982). However, a change in the proportionality of either host, pathogen or environmental components may alter the 'steady state' of the relationship and result in increased disease incidence (McVicar, 1990). Periods of both chronic and acute stress of fish populations within the culture environment have physiological effects that are sufficient to skew the balance to the extent that major epizootics began to occur during the rapid expansion of the Scottish salmon farming industry in the 1980's (Ellis, 1981). It was only following on from these outbreaks that intensive investigation into sea lice biology and population dynamics began, which gave an insight into sea lice as a disease of farmed fish.

In common with other members of the family Caligidae, *L. salmonis* has a 10 stage lifecycle comprising 3 planktonic and seven attached stages (Kabata, 1973; Boxshall, 1974; Pike, 1989; Johnson & Albright, 1991; Schram, 1993). The female produces a pair of eggstrings containing up to 700 eggs (Wootten, *et al.*, 1982), which are initially opaque but become black as they mature and hatch to the first of 2 nauplius stages (Wootten, *et al.*, 1982; Johnson and Albright, 1991). All larval stages are free-living, positively phototaxic and go through cycles of active upwards swimming followed by passive sinking (Wootten, *et al.*, 1982; Heuch, 1995; Heuch *et al.*, 1995). In the infective copepodid stage, which follows nauplius II, such behaviour has been associated with host finding (Bron *et al.*, 1991; Bron *et al.*, 1993; Heuch, *et al.*, 1995). The copepodid stage is thought to exhibit host-finding behaviour for up to 4 days, although is reported to survive for up to 1 month post metamorphosis (Johannessen, 1975; Hastein & Bergsjo, 1976). Bron *et al.* (1991) suggest 3 phases of settlement and attachment following contact with a potential host. On first contact with the fish the copepodid uses the maxillipeds to grip the surface. This allows movement over a small area of the surface, which is probed with the anterior cephalothorax, and first and second antennae. These possess a large

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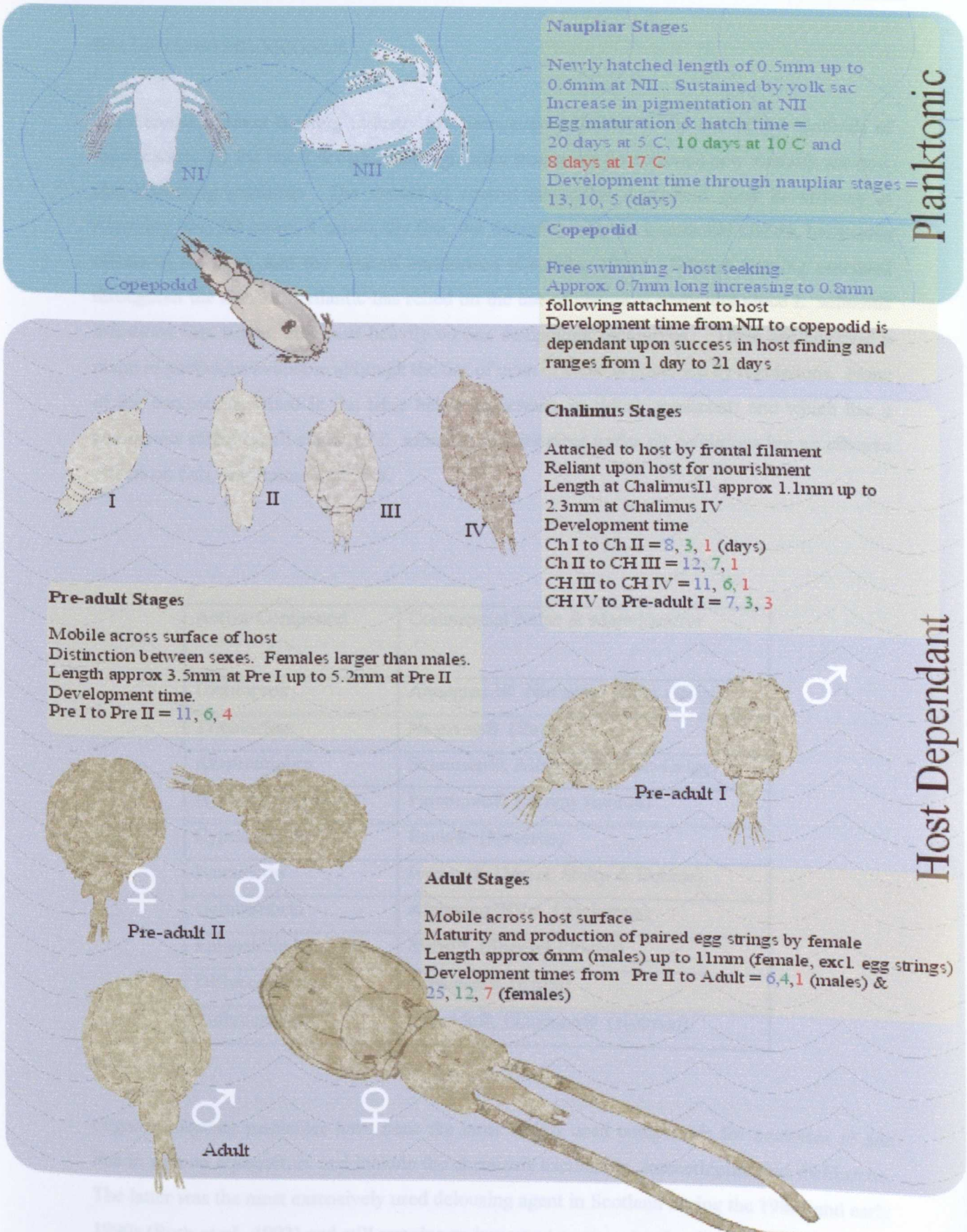

Figure 1.1

The life cycle of *L. salmonis* indicating the approximate developmental time (days) between successive stages at 17°C (highlighted in red), 10°C (green) and 5°C (blue). The diagrams of louse morphology shown are not to scale, and are modified from figures published by Schram (1993). The developmental times are taken from work by Wadsworth *et al* (1998), and from unpublished personal observations

SEA LICE CONTROL METHODS

The Scottish salmon farming industry has been largely limited to the use of 2-3 methods of louse control for the last 2 decades although other treatments do exist and new methods are now also becoming available. The choice of control method is dependent upon its efficacy in removing lice, the stress it causes the fish, the financial cost, environmental effects, hazardous effects to the user, and the ease of application (Costello, 1993). Salmon farming practised throughout the northern Atlantic has relied on the use of chemical pesticides since *L. salmonis* infections first began, and most heavily on one compound; dichlorvos. There are however a range of pesticides available although the use of most of these is governed by regulations. None of the compounds listed in the table below represents an 'ideal' treatment: one which has a continuous effect on all stages of *L. salmonis* development under all conditions but no adverse effects on fish, environment or user.

Active Compound	Commercial Name & Manufacturer
Dichlorvos	Aquaguard®, Nuvan® (Ciba-Geigy)
Trichlorfon	Neguvon® (Bayer)
Azamethiphos	Salmosan®, Alfacron® (Ciba-Geigy)
Hydrogen peroxide	Paramove® (Solvay Interox)
Cypermethrin	Excis® (Novartis)
Ivermectin	Ivomec® (Merck Sharp & Dohme)
Deltamethrin	Alphamax™Vet (Alpharma)
Emamectin benzoate	Slice™ (Shering-Plough)
Diflubenzuron	Lepsidon® (Ewos)
Teflubezuron	Calicide®, Ektobann® (Nutreco)

Organophosphate pesticides have been the most widely used compounds for treatment of sea lice in salmon aquaculture and include the chemicals trichlorfon, azamethiphos and dichlorvos. The latter was the most extensively used delousing agent in Scotland during the 1980s and early 1990s (Roth *et al.*, 1993) and still remains an important treatment today (Rae, 1997). However, it is a substance 'red listed' by the North Sea Conference and its use is restricted by the Scottish

Environmental Protection Agency (SEPA)(Rae, 1997). It is efficacious against only the mobile stages of *L. salmonis* when administered as a bath treatment (Rae, 1979; Wootten, *et al.*, 1982; Pike, 1989; Roth *et al.*, 1993) and so repeated treatments are required in order to be effective against the larval stages developing through to adult. Ross (1989) makes the point that the environmental and ecotoxicological risks from the use of dichlorvos in the marine environment appear to be a perceived rather than an actual problem. Dichlorvos is released directly into the sea following use but is rapidly dispersed (Dobson & Tack, 1991) and is biodegradable (Roth, *et al.*, 1993). In *in vitro* bioassays it appears toxic to larval lobster (*Homarus gammarus*) following repeated exposure (Cussack & Johnson, 1990; McHenery *et al.*, 1991; McHenery *et al.*, 1996), but in field trials no toxic effects were observed when lobster larvae were suspended in the water column in the vicinity of treatment cages (McHenery, 1990). McHenery *et al.* (1990) found no observable effects on mussel (*Mytilus edulis*) mortality *in vitro*, and Murison *et al.* (1990) report that dichlorvos treatments do not deter settlement and growth of mussel larvae around salmon cages in Scotland. Whilst sub-lethal effects on tropical freshwater fish have been observed (*Oreochromis mossambicus*)(Roth, *et al.*, 1993), no similar conditions are reported in marine cultured salmon when dichlorvos is applied at the recommended commercial dosage (Dobson & Schuurman, 1990). However, the major problem of dichlorvos is its efficacy, especially given the observations of many salmon farmers and the experimental evidence of Jones *et al.* (1992) which showed reduced sensitivity of *L. salmonis* to the compound. The apparent development of resistant strains of lice may force increases in dose rates and in doing so reduce the safety margins to fish and to the handler. The mode of toxicity of this and other organophosphates is its inhibition of cholinesterase at neural synapses (Baillie, 1985) which makes it extremely hazardous to mammalian users of the compound (Worthing & Walker, 1987).

Trichlorfon was developed and used as a sea lice treatment in Norway where it was initially used as an oral treatment (Brandal & Egidius, 1977). However, following side effects such as blindness it was applied as a bath treatment (Brandal & Egidius, 1979). Trichlorfon breaks down to dichlorvos in water at rates which are dependent upon both temperature and pH (Samuelsen, 1987), and since dichlorvos is a much more toxic compound, sporadic fish kills occurred. Its instability in water and variable degradation rates meant that trichlorfon was largely superseded by the use of dichlorvos.

Azamethiphos is available under the trade name Salmosan® and was developed by the manufacteuers of Nuvan® (dichlorvos)(Ciba-Geigy) as a replacement for the compound. However, like its predecessor it is only effective against mobile stages and appears selectively

more toxic towards pre-adult I males (Roth *et al.*, 1996). It is reported to be marginally less toxic than dichlorvos to marine molluscs (Roth, *et al.*, 1993) and effective against sea lice when applied at dose rates lower than dichlorvos (Roth, *et al.*, 1996). Worthing and Walker (1987) also report that it is around ten times less toxic to mammals and so confers a significant advantage with respect to handling. However, cross-resistance between organophosphorus compounds has been reported in insect populations (Kuwahara, 1986; Wirth *et al.*, 1987; Wirth, 1998) and so it is possible that the reduced sensitivity of lice populations to dichlorvos may confer upon them similar resistance to azamethiphos. Roth *et al* (1996) however, found that it was effective against dichlorvos resistant lice populations. Even if cross-resistant populations do not occur, Brown (1986) estimates that, certainly in terrestrial culture environments, parasite resistance to repeatedly used compounds can develop with 2 to 10 years of use.

In recent years hydrogen peroxide, previously used as a treatment of ectoparasites of tropical fish (Kabata, 1985), has been applied as a bath treatment for sea lice on Scottish salmon farms. However, whilst it is effective in removing the mobile stages of *L. salmonis* it is ineffective against the attached larval stages (Roth, *et al.*, 1993; Bruno & Raynard, 1994; Treasurer & Grant, 1997). Johnson *et al.* (1993) do though report a drop on the viability of the eggs of treated females, which may reduce larval recruitment and re-infection rates. The chemical is a strong oxidising agent and quickly degrades to water and oxygen and so has a low environmental impact. Thomassen (1993) suggests that it induces mechanical paralysis owing to the formation of gas bubbles in the haemolymph, which Bruno and Raynard (1994) suggested caused the affected lice to float to the surface. A more biochemical mode of action is described by Cotran *et al.* (1989) whereby hydroxyl radicals may lead to peroxidation of lipids in cellular and organelle membranes, inactivation of enzymes and disruption of DNA replication. The effects on sea lice however appear to be sub-lethal and between 50% and 97% of treated lice recover within 24 hours (Hodneland *et al.*, 1993; Thomassen, 1993; Treasurer and Grant, 1997). The possibility of re-infection by this displaced population therefore exists, although it has not been demonstrated experimentally and is thought by many authors to be unlikely to contribute significantly to future lice infections (Johnson, *et al.*, 1993; Thomassen, 1993; Treasurer and Grant, 1997). Additional drawbacks on the use of hydrogen peroxide are its hazards to human health, toxicity to salmon at higher water temperatures (Costello, 1993; Bruno and Raynard, 1994; Rae, 1997) and recent findings by Treasurer *et al.* (1999) of resistant lice populations. In this study the removal of mobile lice at salmon farm sites previously untreated with hydrogen peroxide was ~95% compared to ~34% on sites where the treatment had been applied on 41 previous occasions.

Cypermethrin is a synthetic pyrethroid, which has recently been permitted use as a treatment for sea lice in Scotland, albeit with strict SEPA regulation. It is effective against the parasite both on contact and when ingested and as well as exhibiting anti-feeding properties its primary mode of action is on basal ganglia of the nervous system, where it causes repeated nervous stimulation through prolongation of sodium permeability of neurones. It is efficacious against adult and pre-adult lice but also targets chalimus larvae, removing 83% following a single exposure as a bath treatment (Wadsworth *et al.*, 1999). Its efficacy within commercial salmon operations is yet to be determined.

Bath treatments with the chemicals described above are technically difficult to perform, are labour intensive and restricted by weather and tidal conditions. Research into chemotherapeutants that are deliverable orally has produced several compounds which can be mixed with fish feed with no reduction in palatability and which are safe to administer and handle. These compounds have only recently been allowed to be applied with commercial situations, but extensive experimental data and initial reported from the field are encouraging.

Ivermectin is a member of the avermectin group of compounds, which are neurotoxins that have been used successfully in treating helminth and arthropod parasites in agriculture (Campbell, 1989). Its efficacy against parasites of fish was first explored with sculpin (*Cottis bairdi*) infected with nematodes (Heckmann unpublished data in Palmer *et al*, 1987). Experimental evaluation for use against *L. salmonis* in Atlantic salmon was first performed in Ireland in the mid 1980s (Palmer, *et al.*, 1987) where lice numbers were significantly reduced following a single treatment (1 day). When administered at days 3, 17 and 39 the experimental population remained free from infection for 60 days. Ivermectin is effective against all developmental stages of *L. salmonis,* although treatment may not cause prompt death of the parasite (Palmer, *et al.*, 1987). When used against ticks Campbell (1989) also reports that death is not immediate, rather the compound disrupts essential processes such as moulting, feeding and reproduction. Insufficient data concerning its effects on non-target organisms, degradation rates in the marine environment, toxic effects on salmon and bioaccumulation within salmon tissues prevented its licensing for use on Scottish fish farms. However, reports of illegal use were widespread, which quickly prompted the necessary research (Spencer, 1992; Roth, *et al.*, 1993). Johnson *et al.* (1993) reported that Atlantic salmon were less tolerant of orally administered ivermectin than either coho salmon or chinook salmon. This study also demonstrated the narrow safety margins of the drug; the normal dose rate of 0.05mg/kg biomass every third day when given every 2 days caused 10% mortalities in the Atlantic salmon population. The related compound abamectin was found to be highly toxic to the mysid shrimp *Mysidopsis bahia* (Wislocki *et al.*,

1989), however, Davies *et al.* (1997) found that there were no direct toxic effects of ivermectin on another mysid shrimp, *Neomysis integer*. Further studies also showed low toxicity towards the shrimp, *Crangon septemspinosa* (Burridge & Haya, 1993) and a low potential for bioaccumulation by *Mytilus edulis* (Davies, *et al.*, 1997). Wislocki *et al.* (1989) report that ivermectin is resistant to hydrolysis and has a long half life in water, however, since it was also found to bind tightly to sediments below marine cages (Halley *et al.*, 1989) it is unlikely to remain in the water column for long. However, accumulation of the compound in sediments presents its most significant draw back since experimental evidence shows profound lethal and sub-lethal effects on sediment dwelling organisms and communities (Davies *et al.*, 1997; Thain *et al.*, 1997; Davies *et al.*, 1998). Currently permission to use ivermectin in Scotland is granted only in defined geographical locations and with the approval and within the regulations of the SEPA.

Emamectin benzoate is a pesticide used extensively in agriculture to treat arthropod infections of food crops, and is undergoing extensive laboratory and field evaluation as a treatment of sea lice. It is from the avermectin class of antiparasitic drugs which also includes ivermectin, although its mode of action is better understood than that of ivermectin. Emamectin benzoate increases the potential of neurotransmitters like glutamate and gamma-amino butyric acid (GABA) to stimulate the influx of chloride ions into nerve cells that results in loss of cell function, disruption of nerve impulses and paralysis (Baille, 1985). It is effective against all stages of *L. salmonis* and when administered orally at 50µg/kg biomass/day for 7 consecutive days it removes >90% of the louse population within 21 days, which is sustained for up to 10 weeks (Endris *et al.*, 1999; Stone *et al.*, 1999; Stone *et al.*, 2000). Published data of toxicity to salmon and non-target organisms are not yet available, although the manufacturers claim there is no mortality or adverse effects to treated fish or pelagic and benthic organisms (Endris *et al.*, 1999).

Diflubenzuron and teflubenzuron are insect growth regulators that act by inhibiting chitin synthesis and so disrupting cuticular development during metamorphosis (Baille, 1985; Roth, *et al.*, 1993). Diflubenzuron was initially evaluated by Hoy and Horsberg (1991) who reported significant reductions in all parasitic stages of *L. salmonis* when administered at 75mg/kg biomass /day for 14 days.(Hoy & Horsberg, 1991) Teflubenzuron has been shown to be similarly effective against larval and adult lice when fed at 10mg/kg/day for 7 days (Branson *et al.*, 1999). Both compounds have undergone extensive laboratory and field trials and are currently available for use in both Norway and Scotland. Little published data are available on

the environmental safety of these compounds, although Toneby *et al.* (1991) do report accumulation of diflubenzuron in sediments beneath salmon cages, however, no adverse effects on the fauna within these sediments were detected. Erdal *et al.* (1999) also showed that there is low accumulation in salmon tissues with the majority excreted via bile and faeces within 21 days of the treatment period. A statement made by the manufacturer of Calicide/Ektobann (Teflubenzuron) (Nutreco / Trouw (UK), 1998) reads:

"When used as an oral feed for sea lice control it is safe to people, fish, birds, marine-mammals, shellfish and algae.....[teflubenzuron] has been shown to be non-toxic to marine shellfish (mussels) and shellfish embryos (oysters). Teflubenzuron does not bioaccumulate in mussels".

Alternative strategies to the use of chemical pesticides have been evaluated over many years, such as disorientation of lice by light traps (since they are positively phototaxic), or repelling by sound or electric shock, however, these are not commonly used in Scotland (Rae, 1997). Bron *et al.* (1993) has demonstrated that fallowing of marine sites between production cycles has the effect of reducing the rate of re-population of the sites by lice for several months, which reduces the need for chemotherapy. The separation of year classes and fallowing of sites for reasons of disease and environmental management is now common practice amongst the larger salmon producers although is not a realistic management tool for smaller operators (Grant & Treasurer, 1993). The use of biological controls of sea lice populations has been investigated for many years, especially concerning the use of wrasse as cleaner fish (Costello & Bjordal, 1990; Bjordal, 1991; Costello, 1993; Treasurer, 1993; Deady *et al.*, 1995; Tully *et al.*, 1996). The maintenance of goldsinny (*Ctenolabrus rupestris*), rockcook (*C. exoletus*), corkwing (*Crenilabrus melops*) and cuckoo wrasse (*Labrus ossifagus*) in salmon cages has rapidly expanded in Norwegian aquaculture from the total use of 100 wrasse in 1988 up to 3.5 million animals used in 1997 (Kvenseth, 1997). The same author estimates that 50% of all Norwegian farms stock wrasse with their smolts, usually at 2-5% of the salmon population. The Scottish use is estimated at 150000 per year (J. Treasurer, Marine Harvest McConnell, pers.comm.). Whilst they remove all stages of sea lice, Tully *et al.* (1996) reported that they failed to prevent a rising population and severe infestation of salmon smolts by *Caligus elongatus*. The use of this form of biological control has the advantages of providing continuous protection throughout the marine production cycle, having no ecotoxicological impact, being less labour intensive than repeated chemical treatments, and is therefore cheaper than other methods. Their use has no reported adverse effects on salmon although mortalities attributed to *Aeromonas salmonicida* are documented (Treasurer & Cox, 1991; Treasurer & Laidler, 1994; Bricknell *et al.*, 1996), as is the recovery of Infectious Pancreatic Necrosis Virus (IPNV) following experimental infection

of goldsinny wrasse (Gibson *et al.*, 1998). These organisms are both known pathogens of Atlantic salmon and may present a health risk to the farmed population. However, Treasurer and Laidler (1994) were unable to detect carriers of *A. salmonicida* in a recovered population of wrasse. They also noted that titres of specific antibody against the bacteria were elevated following vaccination with a commercial vaccine for salmon, they did not however go on to demonstrate protective effects of the increased antibody titre. Currently those Scottish farms stocking wrasse still continue to employ chemical pesticides as their principal control method.

Vaccination of salmon against sea lice is potentially the most valuable method for their control. However, since natural infections of *L. salmonis* fail to stimulate specific antibody responses by parasitised fish it is evident that a novel approach is required to identify potential louse antigens. Several groups have been involved in research to identify 'hidden' antigens that in the normal course of an infection are not exposed to the hosts immune system. This follows work by Willadsen (1987), Wikel (1988), Willadsen *et al.* (1989), Willadsen and McKenna (1991) and Lee *et al.* (1991) who successfully vaccinated cattle against the tick , *Boophilus microplus*, using antigens from the parasites gut. Similar investigations have been conducted using *L. salmonis* (Grayson *et al.*, 1991; Jenkins *et al.*, 1993; Jenkins *et al.*, 1994; Raynard *et al.*,1994 ; Grayson *et al.*, 1995; Roper *et al.*, 1995) with varying degrees of success. None of those involved have yet identified protective antigens although Grayson *et al.* (1995) report a reduction in the number of lice becoming egg-bearing on immunised fish and also that those that were gravid produced fewer eggs. At this time there are no published reports of protective antigens, however their identification and subsequent incorporation into an effective vaccine is a difficult and labour intensive process which may take many more years. However, the rationale behind the current vaccine studies may be flawed. Sea lice have been seen to consume blood as part of their diet (Voth, 1972; Johannessen, 1975; Brandal *et al.*, 1976), however, mucus and epithelial cells are considered to be the primary food source (Voth, 1972; Wootten, *et al.*, 1982; Pike, 1989). The primary food source of *B. microplus*, the tick whose successful vaccination strategy forms the basis of the current research into sea lice vaccines, is blood (Wikel, 1988). Furthermore, ticks gorge themselves on host blood and then may fall from the host before slowly digesting the meal for up to 24 hours. *L. salmonis* do not appear to gorge feed and the rate of passage of food through the gut certainly appears faster than that of the tick (pers obs.). If this is the case then the residence time of salmon antibodies from a possible blood meal in the gut of *L. salmonis* may be insufficient to permit their effective function. In addition, the biochemical environment of the louse gut is not yet fully understood and it is certainly plausible to consider that the conditions may not be conducive to antibody survival or activity.

The production of a louse vaccine would end the requirement for widespread chemical treatments which would be beneficial to both fish and fish framer, however, it is important that research is not focussed on only one strategy. It is clear though that vaccination as a means of controlling sea lice is a distant prospect, and for the time being the use of chemical pesticides is the most effective protection from damage and death due to *L. salmonis* infestations of farmed salmon.

CULTURE OF FISH CELLS

A glossary of terms is included in Appendix 1 of this thesis

Tissue culture, the generic term that includes cell, tissue and organ culture, was first devised at the start of the twentieth century using the frog as a tissue donor (Harrison, 1907). Similar early studies allowed the short-term culture of fish cells on slides or watchglasses using physiological salt solutions and frog lymph (Osowski, 1914), or seawater, dextrose and fish bouillon (Lewis, 1916) as culture media. However, the comparative ease of mammalian tissue culture and the obvious potential applications in human medical research meant that lower vertebrates and invertebrates were largely overlooked (Freshney, 1998).

Advances in the 1950's however paved the way for other research and rekindled the interest in the cultivation of fish cells. Sanders and Soret (1954) were the first to successfully grow a virus (Eastern Equine Encephalomyelitis Virus) in the cells of the Eastern Mosquitofish, *Gambusia holbrookii*, and to use a synthetic medium, Medium 199, designed for mammalian cell culture, whilst Grutzner (1958) showed the use of trypsin to dissociate cells from tissues of the tench, *Tinca vulgaris*, which subsequently grew as a monolayer. The same author is also credited as being the first to culture a fish virus (Lymphocystis virus) in fish cells (Grutzner, 1956). Clem *et al.* (1961) were the first to report an established cell line from a marine teleost, the reef fish *Haemulon sciurus*, bluestriped grunt. Subsequent cell lines have predominately been developed from commercially important species, both fresh water and marine, and were reviewed by Wolf and Mann (1980) with additions being made by Hightower and Renfro (1988). An up to date review remains thus far unpublished, but is likely to include at least a total of 90 established teleost lines.

The fishes comprise 48% of the known vertebrate species (Altman & Dittmer, 1972), which represents an enormous resource for the development of vertebrate cell and tissue models for use in the biomedical sciences. Hightower and Renfro (1988) review current applications of fish cell lines in the study of intracellular movement of organelles, xenobiotic metabolism, thermal stress, ion transport, endocrinology, immunology and cancer biology. However, much of the interest continues to be centred on their use in monitoring aquatic pollution and toxicology, and in propagating viruses that are etiologic agents of disease in economically significant species. The distribution of fish cell lines across the piscean classes is therefore greatly biased towards commercially important food and sport fish, such as members of the Salmonidae, which account for two-thirds of existing lines (Hightower and Renfro, 1988).

Culture of cells *in vitro* has two major advantages (Freshney, 1998). Firstly it provides a controlled environment where pH, temperature, osmotic pressure and gas tensions can be precisely defined and kept constant, and secondly it ensures homogeneity of samples. Tissue samples are invariably heterogeneous, and replicates of the same tissue produce varied responses. The selective pressures of the culture conditions tends to produce a homogeneous culture of a single, vigorous cell type. Since experimental replicates are virtually identical the need for statistical analysis of variance is reduced. A significant disadvantage though is the loss of differentiated cell properties since standard culture environments are designed to primarily encourage cell proliferation. The resultant cell phenotype differs dramatically with increasing passage from the characteristics of the donor tissue. In consequence, these cells may have different morphology, biochemistry and function from their counter-parts *in vivo*. Nevertheless established monolayer cultures of single cell types form the basis for the majority of tissue culture research. Mammalian cells have inevitably been the most studied and Freshney (1998) summarises their use in 6 areas of biomedical investigation; (1) Intracellular activity, DNA replication and transcription, protein synthesis, energy metabolism, drug metabolism; (2) Intracellular flux, RNA, translocation of hormone receptor complexes, membrane trafficking; (3) Environmental interaction, nutrition, infection, carcinogenesis, drug interaction; (4) Cell-cell interaction, cell population kinetics, cell adhesion, cell invasion, embryonic induction; (5) Cellular production and secretion; (6) Genetics, genetic manipulation, transformation and immortalization.

However, cell monolayers cannot accurately reproduce the responses of the original tissue the research may wish to study, for instance, they lack the potential for cell-cell interaction and cell-matrix interactions which participate in the desired whole tissue response *in vivo*. For these reasons much of this work is now performed using high-density aggregated cell suspensions

reconstituted on either artificial or biological substrates to form three-dimensional cellular structures. The use of histotypic ('tissue-like' culture of single cell type) and organotypic (organ-like assemblage of several cell types) cultures has brought about significant advances in the understanding of cell biology, and led to the growth in the new science and commercial possibilities of tissue engineering.

TISSUE ENGINEERING

Tissue engineering is a cross-disciplinary field combining expertise from biochemistry, cell and molecular biology, genetics, biomedical engineering and materials science (Schwab & Isseroff, 2000). The science is currently focused on the development of viable substitutes which restore, maintain or improve the functioning of human issues and which have direct clinical applications. There are three general approaches to the creation of new tissues (Arnst & Carey, 1998). The first is the implantation of cellular devices which stimulate the regeneration of functional tissue by the patients body. This includes biomatrices used to promote bone re-growth following periodontal disease or maxileofacial surgery, and skin equivalents which provide wound coverings that promote the proliferation of dermal and epidermal tissue in areas of trauma that would not normally regenerate. The next approach is the design and growth of human tissue outside of the body for later re-implantation to repair or replace diseased or damaged tissues. The use of skin equivalents also fits into this category when used to replace skin in areas of injury where no regeneration is possible. The final approach is the development of devices containing human tissue that will totally replace the function of diseased organs. This area of research is still very much in its infancy although significant advances have been made towards the production of bio-engineered livers and hearts. Biotechnology companies and researchers are currently in various stages of development of engineered body parts as replacements for bone (Whang *et al.*, 1999), cartilage (Peretti *et al.*, 1999), ligaments (Lin *et al.*, 1999), salivary glands (Aframian *et al.*, 2000), blood vessels (Kobashi & Matsuda, 1999a; Kobashi & Matsuda, 1999b), nerves (Hadlock *et al.*, 2000), heart (Hoerstrup *et al.*, 2000a; Hoerstrup *et al.*, 2000b; Sodian *et al.*, 2000a; Sodian *et al.*, 2000b), pancreas (Papas *et al.*, 1999; Tziampazis & Sambanis, 1995; Tziampazis & Sambanis, 1996)and liver (Kaihara *et al.*, 2000). Many of these tissues are undergoing clinical trials but the development of functional replacement heart and livers is thought to be several decades away (Arnst and Carey, 1998). However, the first wholly bio-engineered tissue to gain a commercial product license is the Living Skin Equivalent (LSE) first described by Bell *et al.* (1981a) which was approved for the

treatment of venous leg ulcers by the American Food and Drug Administration in May 1998 under the product name Apligraf® (Organogenesis Inc., USA).

THE STRUCTURE OF THE SKIN OF TERRESTRIAL VERTEBRATES

The basic cellular components of vertebrate skin are the epithelial cell, also referred to as the Malpighian cell or keratinocyte, in the epidermis, and the fibroblast in the dermis. These cell types, their association and patterns of aggregation as well as metabolism result in the clear formation of two principal layers of the skin, the epidermis and the dermis (Green *et al.*, 1989). Figure 1.2 displays diagrammatically the strata of mammalian skin which will be described below.

The structure of the epidermis is maintained by intercellular junctions which are located laterally (desmosomes) that connect adjacent cells, and basally (hemidesmosomes) which form strong cohesion between cells of the lower epidermis and the basement membrane (Shaw, 1996). The extent of desmosomal connection is one factor distinguishing the layers of the epidermis, with close association in the basal and surface layers, and less structural association in the mid layers. However, cytoplasmic architecture and mitotic activity are the greater determinants of epidermal strata formation. Cytokeratins are intermediate filaments (10nm) found in epithelial cytoplasm. Around 20 have been identified, which is a complexity unusual by comparison to the number of intermediate filaments of other animal cell types (MacKenzie & Gao, 1996). They exist in both acidic and basic forms, each denoted by K↓, and the formation of keratin filaments requires the heteropolymerisation of the two forms. All epithelia therefore possesss at least two cytokeratins (Moll *et al.*, 1982). Keratinocytes undergo keratinisation, a process of differentiation with terminal differentiation being achieved in the outer most epidermal layer. As keratinocytes are displaced upwards through the epidermis by cell division in the basal layers, cytokeratins begin to aggregate to form filaments, and with further ribosomal processing they form larger filament groups (tonofilaments and tonofibrils) (Smack *et al.*, 1994). Cells of the mid epidermis also contain keratohyalin granules which are composed of filaggrin and loricin; the first is responsible for aggregation and parallel orientation of tonofibrils and the second is involved in the cross-linking of tonofibrils to the cell membrane of cells undergoing keratinisation (Shaw, 1996). Keratinocyte stem cells possesss approximately 30% cytoplasmic keratin, but a fully differentiated keratinocyte is anuclear, has no cell organelles and is composed of 85% keratin (Smack, *et al.*, 1994).

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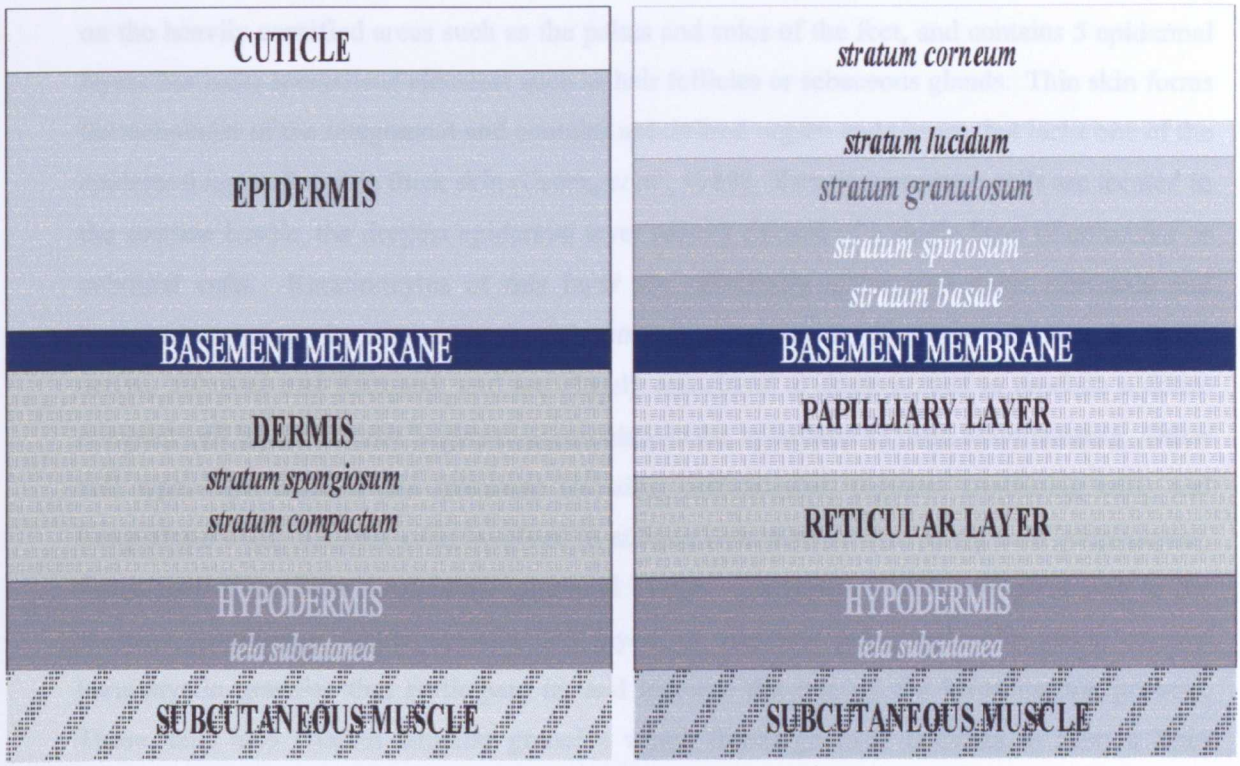

Figure 1.2

Schematic diagram of the comparative anatomy of normal mammalian skin (right) and normal marine teleost skin (left) showing the structural composition of each and the apparent additional complexity of mammalian epidermis.

The associated metabolic and morphological changes involved in keratinocyte differentiation further divides the epidermis of the majority of terrestrial vertebrates into either 4 or 5 layers (Green, *et al.*, 1989). In mammals, skin is described as being thick or thin. Thick skin is found on the heavily cornified areas such as the palms and soles of the feet, and contains 5 epidermal layers but lacks specialised elements such as hair follicles or sebaceous glands. Thin skin forms the remainder of the integument and contains specialised organs and tissues, but lacks one of the epidermal layers found in thick skin (Green, *et al.*, 1989). Keratinocyte stem cells are located in the *stratum basale*, the deepest epidermal layer usually formed of a single layer of columnar or cuboidal cells. Keratinocytes of this layer are mitotically active and move outwards and become increasingly keratinized to form the *stratum spinosum*. In this layer cells are mitotically active, cuboidal and flattened, and are closely associated at desmosomes to produce a dense compact cell layer. Keratin begins to accumulate in the cytoplasm to form tonofilaments. Other cell types also exist in the *spinosum* including melanocytes, pigment containing cells and Langerhans cells, immune cells involved in antigen presentation that, along with lymphocytes, forms part of the Skin Associated Lymphoid Tissue. The *stratum spinosum* gives way to the *stratum granulosum* which contains 3-5 layers of flattened polygonal cells which contain keratohyalin granules that participate in, and increase the rate of, the keratinisation process. These cells also contain lamellar granules which discharge their contents to form a lipid 'cement' between cells in this and subsequent layers. These cells do not undergo mitosis but do possess organelles and a nucleus which are absent in the remaining strata. The *stratum lucidum*, not present in mammalian thin skin, is a discrete layer of flattened eosinophilic keratinocytes that possess no cell organelles or nucleus, but are rich in eleidin, a component of keratin synthesis. Cells in this layer are not mitotically active and demonstrate the first indicators of degradation and cornification. The *stratum corneum* is a layer of aggregated fully keratinized epithelia with between 15-30 cell layers. Cells are flattened, anuclear and non-living, and are composed primarily of keratin. The integrity of this layer is maintained by intercellular connection at desmosomes and by intercellular lipids discharged by lamellar granules of keratinocytes from the *stratum granulosum*. Dead keratinocytes are continually desquaminating which in normal skin is balanced by the rate of keratinocyte production in the *stratum basale* (Green, *et al.*, 1989; Smack, *et al.*, 1994; Myers *et al.*, 1995; Shaw, 1996).

The basement membrane, separating the epidermis and dermis, has been studied more in the skin of mammals than in any other vertebrate phyla . Structurally it is an acellular layer of extracellular matrix that can be further divided into 3 layers. The *lamina densa* is an electron dense layer running parallel to the basolateral membranes of the epithelia in the *stratum basale*.

It is straddled by electron-lucent layers, the *lamina lucida* on the epidermal side, and the *lamina fibroreticularis* on the dermal side (Shaw, 1996). Its primary function is to provide a flexible anchorage for the two skin layers, the cells of which share few biochemical interactions directly. The *lamina densa* comprises a mesh of type IV collagen, laminin and proteoglycans which have affinity for receptors on the basal surface of epithelial cells. In contrast, the *lamina fibroreticularis* contains type III collagen which is bound by fibronectin to surface receptors on fibroblasts and reticular fibres of the dermis (Green, *et al.*, 1989; Myers, *et al.*, 1995; Shaw, 1996).

The dermal structure is maintained by fibroblasts which are mesenchymal cells responsible for the production of extracellular matrix, primarily type I and type IV collagens, but also elastin, reticulin, glycosaminogylcans and fibronectin (Lamme *et al.*, 1998). They also possess receptors for fibronectin which they aggregate to contribute to the formation of reticular fibres. However, the cellular component of dermis is low with approximately 90% of the volume being composed of collagen fibres which impart strength to the skin (Green, *et al.*, 1989). It is structurally organised into the upper papillary layer and the lower reticular layer. The upper layer forms papillae, convolutions which reach into the main structure of the epidermis and counter shearing forces between the two layers. It also contains capillaries and nerve endings unlike the reticular layer which is an assemblage of dense reticular fibres and larger blood vessels, nerves and lymphatics. Fibroblasts are seeded between fibres throughout the dermis but are less numerous in the reticular layer and they do not aggregate in dense strata that might result in the cellular layering seen in the epidermis (Green, *et al.*, 1989).

THE STRUCTURE OF TELEOST SKIN

The integument of all vertebrates functions primarily as a complex limiting membrane providing mechanical protection against the environment. In the teleost it also serves to preserve and regulate homeostasis by the osmoregulatory capacity of the epidermis and, in larvae, by its respiratory and excretory capacity (Bullock & Roberts, 1974). It also contains inhibitory substances as protection from pathogenic invasion (Fletcher & Grant, 1969; Baldo & Fletcher, 1973; Fletcher & White, 1973; Ellis *et al.*, 1989), venom glands as deterrents to predation (Russell, 1971), pheromones that allow communication between individuals (Bullock

and Roberts, 1974), and possesses neurosensory structures for taste and detection of chemical (Whitear, 1952; Whitear, 1970) and mechanical stimuli (Webb, 1989). The presence of these and other specialised tissues and metabolites in fish skin varies between species, between the sexes, between stages of development and phase of the reproductive cycle (Roberts *et al.*, 1970). However, the basic stratified structure of teleost skin is comparable to that of most vertebrates, except for obvious specialisations such as the presence of scales and the outermost acellular mucoid layer referred to as the "cuticle" (Whitear, 1970). A diagrammatic representation of fish skin can be seen in figure 1.2. The outermost living tissue is the epidermis which is an avascular cell layer overlying the mesenchymal dermis. The dermis contains collagen fibres, providing structural strength, and also scales, pigment cells, nerves and vessels circulating blood and lymph. These upper layers are fixed to subcutaneous muscle by a layer of areolar connective tissue called the hypodermis.

Fish skin possesses an external coat of mucus that, prior to the work of Whitear (1970) was presumed to be secreted by goblet cells contained in the epidermis. There is now though agreement that it is in fact a 'formed' supra-epithelial layer secreted by surface epithelial cells and named the 'cuticle' (Whitear, 1970; Bullock and Roberts, 1974). It differs in its biochemistry to goblet cell derived mucus in being composed mainly of mucopolysaccharide (Whitear, 1970) as opposed to sialic-acid containing glycoprotein reported as the major component of salmonid mucus (Harris, 1974). Its occurrence in salmonids has been described by Roberts *et al.* (1970) and in many other species, both marine and freshwater which are reviewed by Bullock and Roberts (1974). The depth of the cuticle varies between species and between areas of the same fish but ranges from 0.25μm in the minnow, *Phoxinus phoxinus*, up to 50μm in the gurnard, *Trigla lucerna* (Whitear, 1970). The mucoid layer is continually sloughing and by doing so is able to 'trap' micro-organisms to inhibit colonisation of the integument (Ellis, *et al.*, 1989). Furthermore, it has been shown to contain elements of the non-specific immune system including C-reactive protein (Baldo and Fletcher, 1973), lysozyme (Fletcher and White, 1973) and free fatty acids which are suggested as having virucidal activity (Lewis, 1971), as well as specific immunoglobulins (Fletcher, *et al.*, 1969; Ling, *et al.*,1993).

In common with other vertebrates the epithelial cell is the fundamental unit of the epidermis. In most teleost species it is a stratified squamous epithelium but unlike mammalian epidermis it is living and capable of mitotic division at all levels and not restricted to the basal layers as in the epidermis of higher vertebrates (Roberts, *et al.*, 1970). This creates the most obvious difference from mammalian skin, namely the absence of stratification within the epidermis forming the *stratum spinosum*, *stratum granulosum* and *stratum corneum*. In fish, the epithelial cells are

normally similar whether they are found in the basal or mid epidermis (Bullock and Roberts, 1974). However, differentiation is evident in the surface epidermal layers where mucus cells, club cells and granule containing cells, as well as specialised sensory tissues and organs are present (Bullock and Roberts, 1974; Roberts, *et al.*, 1970). For the majority of teleost species there is no cellular keratinisation, the process which leads to the development of *stratum corneum*, the outermost layer of mammalian skin. However, evidence for a weakly keratinized epidermis and cuticle was provided by Bone and Brook (1973) who identified keratinized skin in the medusa-consorting cornish blackfish, *Schedophilus medusophagus*. It is also known that the cuticle and epidermis on the ventral fins of the shanny, *Blennius pholis*, are thickened to provide protection against skin damage within its rock pool environment (Whitear, 1970; Bullock and Roberts, 1974). This leads some scientists to draw analogies between the cuticle of teleosts and the *stratum corneum* of higher vertebrates. The cells at the base of the epidermis usually have a vertical orientation and are closely aligned with their cell membrane contiguous with the basement membrane. As cells pass up through the epidermis, the cytoplasm becomes denser and more fibrous and the cells become horizontally orientated and only loosely associated. Sloughing cells at the surface of the epidermis consist only of a cytoskeleton and possesss few cytoplasmic elements but are closely associated at lateral desmosomes to form a 'junctional complex' limiting barrier (Ellis, *et al.*, 1989; Roberts, *et al.*, 1970).

The basement membrane is a complex of collagen fibres and extracellular matrix lying between the epidermis and dermis and, in teleosts, is relatively thick at 1μm, or greater when underlying un-scaled skin (Bullock and Roberts, 1974). By contrast, in mammals it is normally less than 100nm thick (Shaw, 1996). Its morphology varies between species, but, in salmonids, it exhibits micro-convolutions and ridges which cause the basal epithelium of the epidermis to have an irregular pattern. These resulting folds are filled by extracellular matrix to give a structural complexity adapted to deal with the shearing processes to which the epidermis is subjected (Roberts, *et al.*, 1970).

The dermis is divided into two layers, *stratum spongiosum* and *stratum compactum*. The *stratum spongiosum* is the upper dermal layer and is contiguous with the basement membrane. It is composed of fibroblasts and a loose network of collagen fibres and contains the scale beds, scale synthesising tissue and chromatophores (Bullock and Roberts, 1974; Roberts, *et al.*, 1970). Scales of teleosts originate in the *stratum spongiosum* and penetrate the basement membrane and epidermis with a posterior orientation. Ultrastructurally, they consist of collagen fibres and a matrix of albuminoid material seeded with hydroxyapatite crystals (Ellis, *et al.*, 1989). The *stratum compactum* consists of a dense collagen matrix with fibres running at right angles to

each other and is responsible for the structural integrity of the skin. The only cellular component is the dermal fibroblast and apart from being penetrated by vascular and neural tissues it is relatively structureless (Bullock and Roberts, 1974; Ellis, *et al.*, 1989).

The hypodermis consists of a fine network of loosely organised connective and adipose tissue. It is a flexible tissue which allows movement of the skin between the *stratum compactum* and the underlying skeletal and muscular elements. In un-scaled areas, such as the head, it is indistinguishable from the dermis (Roberts, *et al.*, 1970; Bullock and Roberts, 1974; Ellis, *et al.*, 1989).

LIVING SKIN EQUIVALENTS

Work towards the creation of human living skin equivalents (LSE), as first described by Bell *et al.* (1981), began in the 1960's with the joint aim of producing functional skin with clinical applications and *in vitro* testing capabilities. The clear driving force however, was the requirement for an artificial skin graft primarily for the treatment of burns, where conventional grafting techniques were not applicable (Arnst and Carey, 1998). Prior to tissue engineered skin, treatment of such injuries was by the application of donated skin; autografts, skin removed from sites on the patients own body; allografts, taken from cadaveric skin ;and porcine xenografts (Eaglstein & Falanga, 1997). Whilst acceptably effective in wound covering and healing the latter 2 grafting techniques carried the risk of immunological rejection and transmission of infectious diseases (Gallico, 1990). However autogeneic, allogeneic and xenogeneic skin grafts did not permit rapid clinical application with predictable outcome, which prompted the search for an alternative that would be non-immunogenic, widely available, and would have properties as close to those of actual skin as possible (Arnst and Carey, 1998).

Modern attempts to develop human skin replacements began in the 1960's but it was the pivotal work by Rheinwald and Green (1975) who succeeded in large scale cultivation of human epidermal cells, which led directly to subsequent tissue engineering successes (Eaglstein and Falanga, 1997). Numerous researchers developed this work to produce the first tissue engineered skin referred to as Expanded Autologous Keratinocyte Grafts (Green *et al.*, 1979; O'Connor & Mullihen, 1981). This technique involved the sub-culture of keratinocytes harvested from a skin biopsy from the patient which, over a period of 3-4 weeks when grown on a feeder layer of irradiated mouse 3T3 fibroblasts, produced a sheet of epidermis. This method

made it possible to cover large areas with sheets grown from a small number of cells. It produced acceptable cosmetic results and its' autologous nature meant that it was not rejected by the patients immune system (Leigh *et al.*, 1991; Eaglstein and Falanga, 1997). Its principal drawback however was the requirement of up to 4 weeks between biopsy and graft. Furthermore, its lack of dermal component meant it lacked mechanical strength, making it difficult to handle. Even though it was immunologically acceptable to the patient, it had only a 60% success rate (Leigh, *et al.*, 1991; Myers, *et al.*, 1995).

However, further work in this area established that serial culture of keratinocytes selects against the proliferation of specialised cells, in particular Langerhans cells (Hefton *et al.*, 1984). These are monocyte derived cells found in the basal layer of skin and which possesses human leucocyte antigens (HLA) that are activators of the immune response. Cultured allogeneic skin was therefore rendered immunologically inert and so showed no signs of graft rejection when applied clinically (Hefton, *et al.*, 1984; Faure *et al.*, 1987). However, it still lacked dermis.

The importance of a dermal component to skin substitutes to control wound contraction and scarring, and to promote rapid healing was recognised early in the development of tissue engineering technology (Gallico, 1990). Early work used acellular bovine collagen matrices to cover wounds (Alloderm®, Life Cell Corp., Texas, USA). These quickly vascularised and formed functional dermal tissue at the graft site and provided physical support both to the wound and to the epidermal graft (either auto- or allogeneic) which was added several weeks later (Gallico, 1990; Eaglstein and Falanga, 1997). This two step procedure and problems of wound drainage limited the use of these products (Nanchahal & Ward, 1992). The progression to cellular matrices using autologous cultured fibroblasts incorporated into collagen-glycosaminoglycan bases initially showed great potential, since the framework allowed rapid ingrowth of fibrovascular tissue (Hansbrough *et al.*, 1989; Yannas *et al.*, 1989). However, in clinical trials the grafts were found to be highly susceptible to bacterial infection and enzymatic degradation which led to graft failure in 70% of cases (Gallico, 1990; Phillips, 1993; Cooper & Spielvogel, 1994; Phillips, 1998). Refinements of this concept however, led to the development of human dermal equivalents such as Dermagraft® (Advanced Tissue Science, Ca, USA) (Gentzkow *et al.*, 1996). This consists of allogeneic human fibroblasts enzymatically isolated from neonatal foreskin and placed in tissue culture. Cells are then seeded onto a polyglactin acid mesh (the same material used in bioabsorbable sutures) and cultured for 3 weeks. The cells proliferate and secrete human dermal collagen, fibronectin and glycosaminoglycans, and growth factors, and produce a dermal extracellular matrix without the requirement for exogenous collagen (Gentzkow, *et al.*, 1996; Eaglstein and Falanga, 1997;). Clinical trials have shown that

dermal equivalents based on this model vascularise quickly at the injury site and following the addition of epidermal grafts, result in complete wound closure within 14 days and the appearance and function of normal skin (Gentzkow, *et al.*, 1996; Hansbrough *et al.*, 1992; Phillips, 1998).

Dermal equivalents however, require the use of a two step procedure; first dermis and then epidermis (Eaglstein and Falanga, 1997). Bell *et al.* (1981a) developed a single organotypic skin model containing both dermal and epidermal components within a biological matrix. They harvested and cultured neonatal foreskin fibroblasts which were then seeded within a bovine collagen lattice which was then further seeded with neonatal foreskin keratinocytes to produce a stratified and organised bilayered skin equivalent in a total manufacturing time of 20 days (Bell *et al.*, 1981a; Bell *et al.*, 1981b; Parenteau *et al.*, 1992; Parenteau, 1994). This technology led to the development of Apligraf® which is the most advanced organ construct to date (Parenteau, 1992; Eaglstein and Falanga, 1997) and which is morphologically, biochemically and metabolically similar to skin (Bilbo *et al.*, 1993). Furthermore, it is immunologically inert due to the absence of Langerhans cells (Bell *et al.*, 1991a).

The validation of LSE as a skin substitute has been undertaken by several authors that have investigated both its biochemistry and morphology (Ponec *et al.*, 1988; Parenteau, *et al.*, 1992; Stoppie *et al.*, 1993; Auger *et al.*, 1995; Black *et al.*, 1998; Ponec *et al.*, 2000). Figure 1.3 presents a summary of the comparisons made by these authors.

The cellular component densities of LSE are approximately $0.4x10^6$ fibroblasts/ml and $1.4x10^6$ keratinocytes/ml with a dermis of 300μm and epidermis of 200μm thick, all falling within the broad ranges of physical constitution of human skin (Parenteau, *et al.*, 1991; Parenteau *et al.*, 1992). Stoppie *et al.* (1993) provide a histological description of LSE that describes a stratified and keratinized epidermis, a basement membrane-like layer and a dense dermis of fibroblasts and extracellular matrix. The focus of this description however is the architecture of the epidermis in which they detail the occurrence of a *stratum basale* of cuboidal cells containing keratin filaments. This is underlaid by a basement membrane in which both a *lamina lucida* and

	In vivo	*In vitro*
	T	T
MORPHOLOGY		
	T	T
Stratum basale		
Stratum spinosum	T	T
Stratum lucidum	T	ς
Stratum granulosum	T	T
Stratum corneum	T	T
Basement membrane	T	Incomplete
Hemidesmosomes	T	T
Desmosomes	T	T
Keratohyaline granules	T	T
Lamellar granules	T	T
FUNCTION		
Physical barrier to xenobiotics	T	Impaired
Metabolism of xenobiotics	T	T
MARKERS OF DIFFERENTIATION		
PROTEINS:		
Cytokeratins (*S. basale*)	K5 & K14	K5, K14 & K19[a]
(*S. spinosum*)	K1 & K10	K1, K10, K6, K16[b] & K19[a]
Filaggrin	ς	T[a]
Involucrin	ς	T[a]
Transglutaminase	*S. granulosum* only	T[a]
LIPIDS:		
Ceramides	T	T
Lanosterol	T	T
Sphinogolipids	T	T
Triglycerides	T	High
Essential fatty acids	T	Low

Figure 1.3

Comparison of characteristics and properties of human epidermis *in vivo,* and Living Skin Equivalent epidermis, *in vitro.* All data are compiled from work by (Ponec *et al.*, 1988; Regnier *et al.*, 1990; Mak *et al.*, 1991; Auger *et al.*, 1995; Ponec *et al.*, 2000).

T Indicates the presence of the substance or property in comparable quantities.
ς Indicates the absence of the substance or property.

[a] K19 is not found in normal human epidermis but is commonly expressed in monolayer cultures of keratinocytes. LSE initially express K19 but this is not found after day 14 of culture indicating the transition to the 'normal' skin phenotype.

[b] K6 & K16 are indicative of hyperplastic epidermis, or skin under-going regeneration and are not normally found in mammalian skin in a 'normal' phase.

lamina densa are evident that are bound by hemidesmosomes on the basal surface of basal keratinocytes. Above the *stratum basale*, the *stratum spinosum* is composed of multiple layers of progressively flattening cells that are tightly associated at desmosomes. The *stratum granulosum* has 3 layers of increasingly keratinized cells containing cytoplasmic keratohyalin granules and the *stratum corneum* is formed of numerous layers of fully differentiated keratinocytes. Continued propagation of the tissue showed persistence of epidermal stratification, an increase in keratohyalin granules and an increase in number and size of hemidesmosomes at the epidermal-dermal junction. They agree with other authors in the conclusion that LSE share many structural and histological features with normal human skin. However, they do show some deviation, for instance in the abundance of lipid droplets and accumulation of glycogen in the cells of the *stratum spinosum*. Further understanding of the biochemistry, cell-cell and cell-matrix interactions is expected to explain the occurrence of these features and hopefully select against their development in future LSE.

Initially LSE were composed of only 2 cell types, keratinocytes and fibroblasts and lacked the additional cell species found in normal skin such as melanocytes or Langerhans cells (Parenteau, 1994). Indeed it is the absence of Langerhans cells which makes LSE successful in grafting procedures since they are not immunologically active and are less susceptible to rejection (Bell *et al.*, 1991b). However, the absence of these cells, as well as specialised tissues such as glands, nerves and blood vessels in *in vitro* models does not allow for a complete understanding of cellular interactions within skin *in vivo*. This is being corrected by several groups who have succeeded in introducing the missing cells types and in forming skin 'appendages' such as hairs, glands and capillaries (Fransson *et al.*, 1997; Michel *et al.*, 1997; Black, *et al.*, 1998; Fransson *et al.*, 1998; Auger *et al.*, 1999; Michel *et al.*, 1999).

Biochemically LSE models exhibit markers consistent with those found in normal human, and indeed bovine skin (Auger, *et al.*, 1995). Metabolically active dermal fibroblasts have been seen to secrete type I, III and IV collagens, fibronectin, and glycosaminoglycans which are observed throughout the dermis and accumulated at the basement membrane (Auger, *et al.*, 1995; Parenteau, *et al.*, 1992). Lipid analysis reveals the occurrence within LSE of all major groups important in the development of a functional *stratum corneum* in human skin, although the relative amounts of these are not always comparable (Ponec, *et al.*, 1988; Ponec, *et al.*, 2000).

The expression of cytokeratins in LSE has been studied by Regnier *et al.* (1990), Parenteau *et al.* (1992) and Auger *et al.* (1995), and all agree on the occurrence of K1 and K10 keratins which are both signs of normal keratinocyte differentiation *in vivo*. Cytokeratins K6 and K16 are also found in LSE but are not usually found in normal human skin. They are classified as hyperproliferative keratins (Regnier *et al.*, 1990) and suggest a continual proliferative state of cultured keratinocytes within skin models. Auger *et al.* (1995) argue that in the clinical situation this would be an advantageous property when applied as a wound dressing, and would increase the rate of wound covering and healing. Cytokeratin K19 is not found in human skin but is a common feature in keratinocyte monolayer cultures (Green, *et al.*, 1979). LSE were initially found to express this protein in the early stages of growth, but it was not observed following 14 days of culture, indicating LSE cellular organisation and the transition from the tissue culture phenotype to the normal skin phenotype (Parenteau, *et al.*, 1992; Parenteau, 1994). Parenteau *et al.* (1992) also described the occurrence of immunological markers as indicators of organotypic functioning within LSE such as β4 integrin, GB3 and heparan-sulphate-proteoglycan which are transmembrane glycoprotein receptors involved in cell-cell, and cell-matrix adhesion, and which are necessary for hemidesmosomal and basement membrane formation .

APPLICATIONS OF LIVING SKIN EQUIVALENTS

Living skin equivalents (LSE) were initially developed as replacement tissue for clinical use but their success in accurately recreating morphologically and biochemically functional skin has led to their application in *in vitro* testing and research. Clinically, LSE have been applied to the treatment of diabetic ulcers, burns, dentistry and cosmetic surgery and, in fact, any surgical procedure or treatment that might have previously required the use of skin grafting (Eaglstein & Falanga, 1997; Eaglstein & Falanga, 1998; Phillips, 1993; Phillips, 1998). However, it is the *in vitro* use of LSE that has seen the broadest range of application in both fundamental research and commercial chemical testing.

Investigations into the suitability of LSE for *in vitro* testing of drugs, cosmetics, consumer products and industrial chemicals came as a result of world-wide consumer-led legislation limiting, and in some cases prohibiting, the use of animals in chemical safety and irritancy testing (Organisation for Economic Co-operation and Development guideline 404, 1992). Research by the European Centre for the Validation of Alternative Methods (ECVAM) (van de Sandt *et al.*, 1997) compared existing animal testing and human cell culture models with *in vivo*

human tissue responses to a range of domestic and industrial chemical irritants. Their recommendations included the standardisation of irritancy and toxicity testing procedures to minimise animal testing, and the use of human LSE where possible. They concluded that LSE are biochemically and morphologically comparable to *in vivo* human skin and that they offered the flexibility in composition, by the addition or subtraction of culture constituents, to enable more thorough understanding of the chemical pathways involved in the skin response to irritants. Furthermore, their use provided a model more suitable for predicting human responses than did the use of animal test subjects, although they did concede that existing LSE were not yet suitable for all types of testing procedures and that animal models still provide the best indicators in these cases. They highlighted advantages of LSE over traditional monolayer cell culture systems, primarily due to the presence of a *stratum corneum* that permits the application of water in-soluble compounds and topical formulations (van de Sandt, *et al.*, 1997; Augustin *et al.*, 1998; Damour *et al.*, 1998).

However, the use of LSE for irritancy testing would require redefinition of assay end points since the principal indicators of irritation in animal testing are erythema and oedema with a range of other effects including blistering and skin dryness. Most of these physical effects do not occur *in vitro* since they are the final physiological manifestations of whole tissue interactions that LSE lack (Kupper, 1990). Therefore, much recent effort has been dedicated to the investigation of morphology, cytokine production and skin physiology markers as more specific *in vitro* irritancy end-points. Morphologically, LSE and human skin are comparable and produce the same responses to chemical irritation including widening of intercellular spaces, disabling of desmosomal connections, cell rounding, nuclear condensation and rearrangement of keratin tonofilaments (van de Sandt, *et al.*, 1997). However, *in vivo,* irritant chemicals cause an influx of inflammatory cells with consequent histological changes which cannot exist in avascular LSE. Whilst LSE can distinguish between irritant and non-irritant compounds by morphological analysis, the degree of irritancy and toxicity is difficult to correlate to that seen *in vivo* using this technique, although a scheme of ranking *in vitro* effects to predicted morphological effects *in vivo* does exist (Parish, 1995; van de Sandt, *et al.*, 1997).

Cytokine production following irritant application is also being studied in LSE models. Cytokines, amongst other chemicals, are responsible for control of inflammatory immune reactions within the skin (Kupper, 1990). The response to skin irritation *in vivo* involves the interaction of resident keratinocytes, fibroblasts and endothelial cells as well as invading leucocytes all under the control of cytokines and lipid mediators (Boss & Kapsenberg, 1993). Keratinocytes produce and retain the cytokine interleukin-1α (IL-1α) which means that the

epidermis is a reservoir of IL-1α that is released following cell damage. This initiates the inflammatory cascade and release of other cytokines, adhesion molecules and chemotaxic factors. Bell *et al.* (1991) identified the presence of IL-1α, amongst other cytokines and inflammatory mediators, in the epidermal layers of LSE. Work by Roguet *et al.* (1994), and Doucet *et al.* (1996) showed the release of IL-1α by LSE keratinocytes following exposure to cutaneous irritation which they were able to correlate to the degree of irritation experienced *in vivo*. Other cytokines, IL-6, IL-8 and TNF-α, have been investigated as markers of *in vitro* toxicity and work is continuing to determine the potential of these to accurately describe the irritancy conditions within LSE to correlate them to the patterns seen *in vivo*.

The use of LSE for the testing of chemical corrosivity to human skin was rated highly in a pre-validation study performed by the ECVAM (Botham *et al.*, 1994). Unlike toxicity and irritancy testing, the endpoint for skin corrosive effects is "....the irreversible full-thickness necrosis of skin after no more than 21 days...."(Botham, *et al.*, 1994) which can be scored easily by visual inspection. In this study 50 chemicals (25 corrosive and 25 non-corrosive) were blind tested by 3 laboratories using both LSE and albino rats (the standard test subject). The animal model achieved between 88% and 98% correct classification, whilst the LSE successfully identified between 64% and 96%. The authors were encouraged by these results and recommended a full evaluation and validation trial in the hope that testers and legislators would realise the potential of LSE for such testing. Commercial chemical testing is currently in the process of using *in vitro* cell culture assays, including LSE, in conjunction with its more traditional animal models for all irritancy and toxicity testing. LSE are therefore now routinely used in the testing of cosmetics (Augustin, *et al.*, 1997; Augustin *et al.*, 1998), sunscreens (Augustin *et al.*, 1997), petroleum products (Koschier *et al.*, 1997), consumer products such as detergents and cleaners (Perkins *et al.*, 1999), soaps and surfactants (Warren *et al.*, 1999) and the delivery of drugs such as antibiotics, fungicides and anti-mycotics (Hanel *et al.*, 1988; Okamata *et al.*, 1989).

In addition to these applied chemical testing and clinical uses, LSE have also been used for more fundamental purposes. The use of human cell culture models for biochemical and physiological studies began in the 1950s (Rinaldini, 1958), with previous data drawn primarily from the culture of rat cells (Waymouth, 1982). Organotypic cultures and explants of human tissue however provide a more representative model of *in vivo* cellular and tissue responses and interactions. Indeed these systems have been used extensively to study liver function, insulin production, hormonal responses, oncogenesis and virology (Freshney, 1998). LSE represent the most advanced organotypic system and their use in research was envisaged by the developers of clinical LSE (Bell, *et al.*, 1981a, 1981b; Bell *et al.*, 1983) who in collaboration with the

biotechnology company Organogenesis Inc. developed an *in vitro* research LSE called Testskin™. This LSE shares the same biochemical and morphological characteristics as the clinical version, Apligraf™. However, whilst Apligraf is formed as a sheet of tissue, Testskin is assembled within plastic multiwell dishes with the obvious practical advantages of handling, maintenance and reproducibility. The use of Testskin and other LSE models within the research environment has obvious benefits over monolayer cell culture however, as a model it still remains very simplistic and does not fully recreate the cellular architecture and associated processes of normal skin. Recent advances in tissue engineering have enabled the culture of additional cell types within LSE and the subsequent formation of specialised tissues such as melanocytes to create pigmented skin (Nakazawa *et al.*, 1997; Nakazawa *et al.*, 1998), endothelial cells to form blood vessels and glands (Black, *et al.*, 1998), dendritic stem cells to create nerves (Konstantinova *et al.*, 1998), and Langerhans cells to provide LSE with an immune system (Fransson, *et al.*, 1997; Fransson, *et al.*, 1998).

Skin equivalents have been extensively used to model pathogenic invasion of the integument by both bacteria and yeast (Bhattacharyya *et al.*, 1998; Phillips, 1998; Rashid *et al.*, 1995). However, few citations exist detailing their use to investigate invasion by parasites. The use of *in vitro* cell culture methods, primarily monoculture systems, is common in the study of intracellular and micro-parasites such as parasitic protozoa like *Toxoplasma gondii* (Chatterton *et al.*, 1999) and *Plasmodium* sp. (Sinnis, 1998), helminths such as *Strongyloides venezuelensis* (Islam *et al.*, 1999) and *Echinococcus multilocularis* (Jura *et al.*, 1996), and filarial nematodes (Cupp *et al.*, 1996). However, such systems are not normally capable of sustaining parasitic organisms throughout the entirety of their life cycle, especially those that require multiple hosts. The use of cell culture models to propagate ectoparasitic organisms is much less common. Recent publications however have detailed the use of cell culture systems for the maintenance and study of the ciliate protozoan fish parasite *Ichthyopthirius multifiliis* (Nielson & Buchmann, 2000) and the monogenean *Gyrodactylus derjavini* (Buchmann *et al.*, 2000). In both studies EPC (Epithelioma Papulosum Cyprini) cells and standard tissue culture media were used. In the case of *G. derjavini* this system enabled parasite survival for up to 139 hours but did not permit development or reproduction. However, the study did succeed in demonstrating the ability of epithelial cells to encapsulate and enzymatically digest gyrodactylids *in vitro* and suggested a role of this tissue in accelerating the elimination of invading parasites *in vivo*.

Nielsen *et al.* (2000) on the other hand, working with *I. multifiliis*, showed that cell culture methods could be used to accelerate development and growth, and increase the survival of the parasites theront and trophont stages. The authors also showed that the inclusion of tissue

culture inserts overgrown by EPC cells, in an attempt at mimicking fish epidermis, allowed superior performance of the parasites in terms of their attachment, survival and growth. This method provided them with a reproducible model that they were able to manipulate to determine the effects of biochemical supplementation on the parasite. To this end they were able to show that the addition of host mucus and serum stimulated parasite development.

Studies involving the use of LSE models for parasitic research do not appear frequently in the scientific literature. In fact this author has been successful in retrieving only a single document detailing such work. Fusco *et al.* (1993) used commercially available human skin equivalent, Testskin™, to investigate its suitability to penetration experiments with *Schistosoma mansoni* cercariae. Cercariae are the infective stage of the Schistosomatidae and previous studies of their penetration through host integument have used dried or fresh animal and human skin, agar, photographic film and extracellular matrix (Fusco, *et al.*, 1993). However, detailed study of cercarial penetration, transformation and cercarial-skin interactions during invasion have been hindered by the lack of biologically relevant *in vitro* models. The authors incubated *S. mansoni* cercariae with Testskin™ and measured the rate of penetration, survival and transformation of the parasite. In normal skin, cercariae can penetrate into the *stratum corneum* within 10 minutes of incubation. In this respect LSE appears to present a more formidable barrier in that only 65% of cercariae were able to penetrate by 3 hours of incubation, and none were observed to penetrate within 15 minutes. Even supplementation of the LSE surface with fatty acids known to induce cercarial proteinase release and allow penetration did not increase penetration through the LSE. Without further morphological and biochemical investigation the authors, prudently, did not suggest that the penetrated cercariae were normal. They did though, despite the differences in the rate and success of penetration, conclude that the use of LSE would provide a suitable biological system to examine penetration and cercarial-keratinocyte interactions without immune cell involvement. However, since this work was performed in 1993 the authors or their colleagues have not, unfortunately, published details of any subsequent studies using LSE.

The lack of suitable biological *in vitro* models for the study of pathogenic organisms is a significant hindrance to many areas of medical and veterinary research (pers. comm. Prof. C. Kennedy). However, it appears that little investment is being made to address this problem and that LSE and other organotypic culture systems are under-utilised in this field of research and seem currently to be used primarily, at least in terms of scale, for *in vitro* chemical testing purposes.

IMMUNOLOGY OF HOST PARASITE INTERACTIONS

In common with other animals, fish are parasitised by a plethora of parasitic organisms from most animal phyla. Research into the host-parasite interactions of these relationships has focused mainly on epidemiological factors of geography, abundance and intensity. However, some parasites cause diseases that result in high mortalities in cultured fish and it is this that has influenced the extensive research into parasitic diseases of fish over the latter part of the twentieth century. Of particular interest is the immunology of parasitic infections both in terms of the immune response of the host and in the biochemical and physiological characteristics and modifications of the infecting organism that permits, or improves the efficiency of its parasitism.

Parasite Mechanisms for Avoidance of the Host Immune Response

Immediately following parasite and host association, the parasite is recognised as foreign and is subjected to a range of immunological defences which transform the host into a hostile environment. Many micro-organisms rely on rapid multiplication to over-power the immune response, however, parasites for the most part, have complex life cycles that require extended periods of development and growth and so they have evolved other ways of evading host defences. These have been most widely studied in mammalian, in particular human disease causing, parasites, but since organisms parasitising both mammalian and piscean hosts are derived from the same phylogenetic families, even genera, it is reasonable to expect that they share common host avoidance mechanisms, even if they have not been scientifically demonstrated (Woo, 1992; Woo, 1997).

Parasitic organisms have evolved 3 general strategies for avoiding host immune responses; immunosuppression at the cellular level; antigenic mimicry; systemic immunosuppression.

Some parasites successfully evade immune responses by inhabiting immunologically privileged sites such as the eye or central nervous system. Others, such as *Trypanosoma cruzi*, *Toxoplasma gondii* and *Leishmania* Sp. inhabit intracellular compartments of host macrophages. Each of these organisms is endocytosed by the macrophage and survives with the phagosome by inhibiting phagosome-lysosome fusion and preventing enzymatic digestion (*T.*

gondii), inhibiting intracellular respiratory burst (*T. cruzi*) or by neutralising oxidative defences by the presence of electron dense outer membranes (*Leishmania* sp.) (Cox, 1984; Cox, 1993).

Another means of evading the immune response is the adoption of host antigens to act as a disguise. This is achieved in several ways including the evolution of parasite surface antigens that cross-react with host antigens, such as murine liver proteins in Schistosomes and human collagen antigens in *Ascaris* sp., which do not permit identification of the parasite as foreign (Turner, 1984). However, the most common disguise is achieved by the acquisition of host antigens at the start of the parasitism, in the way that Schistosomes are able to incorporate red cell A and B glycoproteins, human skin antigens and IgG, and murine MHC products onto their outer surface (Turner, 1984).

As well as passive avoidance methods parasites are also able to attack the immune responses of the host to elicit systemic immunodepression. Studies of the effects of *Trypanosoma brucei*, reviewed by Askonas (1984), report reductions in immune stem cells of 80% and in cell mediated immunity of 90-95% of the normal levels. In this case both the homogenised parasite and its products were immunosuppresive. Schistosomes are also known to actively protect themselves from host immune factors by releasing immunoglobulin cleaving peptidases and inhibitors of T cell proliferation required for eosinophil activation (Langlet *et al.*, 1984), and the production of eicosanoid species that inhibit cellular immunology (Nevutalu *et al.*, 1993; De Jong-Brink, 1995;).

Little is known of the biochemical and immunological modifications of fish parasites other than what has been extrapolated from related parasites in mammalian systems. It is not out of place though to expect them to employ avoidance tactics such as intracellular modification of macrophage functioning and antigenic mimicry in much the same way as other parasites do. Indeed, it is understood from both observation of captive fish populations and from experimental evidence that these parasites do possess mechanisms which result in system immunodepression in the host. For instance, rainbow trout infected by the protozoan *Cryptobia salmositica* show suppressed humoral responses to model antigens, anaemia and increased susceptibility to *Yersinia ruckerii* (Jones *et al.*, 1986; Wehnert & Woo, 1981), and Laudan *et al.* (1986) showed that *Glugea stephani* reduces immunoglobulin levels in the winter flounder , *Pseudopleuronectes americanus*. Systemic immunodepression is also reported in salmonids infected with *Gyrodactylus salaris* (Harris *et al.*, 2000) and PKX, which rendered fish more susceptible to secondary infections (Angelidis *et al.*, 1987).

The evidence of immunosupression of fish caused by arthropod parasites is much less available, and is encapsulated in only a handful of publications. Following observations made by Johnson and Albright (1992) of the apparent correlation between susceptibility and lowered cellular immune responses in infected Atlantic salmon, work by Musfata *et al.* (1999, 2000) has demonstrated systemic immunosupressive effects of *L. salmonis* infection. Johnson and Albright (1992) suggested that the lack of inflammatory responses at the site of parasite attachment was due to localised immunosuppressive effects of the louse. Mustafa *et al.* (2000), somewhat pre-empting the work detailed in chapter 3 of this thesis, expanded upon this finding and showed that the oxidative and phagocytic defence mechanisms of infected Atlantic salmon were significantly reduced following infection. Furthermore, unpublished work by the same authors will show the increased susceptibility of rainbow trout infected with *L. salmonis* to secondary infection by the microsporidian parasite *Loma salmonae*.

Immunological Responses of Fish to Parasitic Organisms

The vertebrate immune system has two functional, interactive divisions; the non-specific (innate) and the specific (adaptive/acquired) systems, each consisting of humoral and cellular components. The piscine immune system is well developed and is normally efficient in protecting healthy individuals from parasitic diseases. However, when fish are cultured in high numbers and stressed by adverse environmental factors, pathological parasitic diseases do occur and it was these instances that both allowed the first studies of the immunology of fish parasitic conditions and prompted increased research into their prevention.

The humoral components of the non-specific immune system identified in fish include: growth inhibitors such as transferrin (Weinberg, 1974) and interferons (Graham & Secombes, 1990); enzyme inhibitors such as α_2 macroglobulin (Ellis, 1987); agglutinins like C-reactive protein (Fletcher *et al* , 1977); and lysins such as lysozyme (Ingram, 1980) and complement (Sakai, 1992). Non-specific cellular immunity is composed of cytotoxic cells and phagocytes. A variety of phagocytes exist in fish; macrophages; monocytes; and neutrophils (granulocytes) (Secombes & Fletcher, 1992) which are involved in the recognition and elimination of foreign cells and damaged tissues. The concentration of these cells at the site of infection is manifested as inflammation, a complex interaction of positive and negative feedback mechanisms controlled by lipid mediators such as eicosanoids, protaglandins, leukotrines and cytokines.

Humoral immunity of the specific immune system is mediated by B lymphocytes and cellular immunity by T lymphocytes and macrophages. The functioning and these cells is reviewed by Lobb and Clem (1982). B lymphocytes express antibodies on their surface which reacts to mitogens on the surface of foreign cells and initiates clonal expansion of B cells and increases the number of specific antibody producing plasma cells (differentiated B cells). The specific antibody interacts with the antigen to form an immune complex which has a variety of functions including fixation of complement and phagocytic opsinisation, both leading to antigen destruction. T lymphocytes complex with antigen and release lymphokine which acts as a chemoattractant that enhances phagocytosis and increases microbial killing. Interactions between the specific immune system and pathogen result in immune memory which constitutes an adaptive change in the lymphocyte population so that invasion by the same antigen is met by a higher magnitude humoral response after a shorter time.

The non-specific arm of the fish immune system is considered a viable target for manipulation for the control of parasitism given its active role in clearing parasitic organisms (Woo, 1992). In particular, complement activation has been shown to be the protective mechanism in many fish against the haemoflagellates *Cryptobia catostomi* (Bower & Woo, 1977) and *C. salmositica* (Wehnert & Woo, 1980), against the cestode *Acanothbothrium quadripartitum* (McVicar & Fletcher, 1970) and against the migrating larvae of the digenean *Diplostomum spathaceum* (Whyte *et al.*, 1989). In many cases of parasitism there is normally an increase in the number of circulating phagocytic cells, however this is not always correlated to protection from the parasite (Woo, 1992; Forward & Woo, 1996). Cellular immunity and tissue inflammation is though implicated in the reduction in egg sac production and population size of the copepod *Ergasilus labriacis* on stripped bass, *Morone saxatilis*, and the reduced susceptibility of coho salmon to infection by *L. salmonis* (Johnson and Albright, 1992).

Specific immunity to parasites is a much studied area given the obvious potential for vaccination against disease. It is known that fish surviving following infection by the dinoflagellate *Amyloodinium ocellatum* (Paperna, 1980), the flagellates *Costia necatrix* (Robertson, 1979) and *I. multifiliis* (Wahli & Meier, 1985), the myxosporean PKX (Clifton-Hadley *et al.*, 1986) and the gill monogenean *Dactylogyrus vastator* (Valdimirov, 1971) are generally protected from disease when later exposed to the same parasite. In each of these cases specific antibody to the parasite was detected, either following primary infection or inoculation with parasite antigens which were protective upon subsequent challenge. Much more is known of specific immunity towards protozoan parasites than metazoan organisms and indeed, prior to the last decade the knowledge of specific immune responses towards arthropod parasites of fish

was particularly scarce. However, it is now known that whilst a specific response was indicated in the resistance of kissing gourami, *Helostoma temmincki*, to infection with *Lernea cyprinacea* (Woo & Shariff, 1990), for the most part specific immunity in fish to arthropod, or at least the well studied crustacean parasites, does not play a significant role in protection. This has been demonstrated notably in *Leiostomus xanthurus* infected with *Lernaeenicus radiatus* (Thoney & Burreson, 1988), in Javanese carp, *Puntius gonionotus*, infected with *Lernaea minuta* (Kularante *et al.*, 1994) and in salmonids naturally infected with parasitic copepods (Grayson, *et al.*, 1991; Mackinnon, 1993; MacKinnon, 1998; Mustafa & MacKinnon, 1999; Ruane *et al.*, 1995). However, specific antibody titres have been shown in salmonids inoculated with louse derived antigens although their protective effects have not been conclusively demonstrated (Jenkins, *et al.*, 1993; Jenkins, *et al.*, 1994; Raynard, *et al.*, 1994; Grayson, *et al.*, 1995; Roper, *et al.*, 1995). These studies were primarily aimed at discovery of potential candidate antigens for a vaccine against *L. salmonis* in Atlantic salmon. The rationale is based on the successful vaccination against the tick *Boophilus microplus* in domestic cattle (Willadsen, 1987; Wikel, 1988; Willadsen, *et al.*, 1989; Willadsen and McKenna, 1991) which was possible because of the extensive scientific information available detailing the immunological interactions between arthropod parasites and their mammalian hosts.

THE IMMUNOLOGY OF HOST AND PARASITIC ARTHROPOD RELATIONSHIPS

The study of arthropod parasites of mammalian hosts has led to vaccination studies in several species based on the understanding of parasite defence mechanisms that permit their parasitism (Wikel, *et al.*, 1996). Modulation of the host immune response by blood feeding arthropods is well documented, although the most extensive body of information on this topic involves the tick (Acari: Ixodidae)-host association (Wikel, *et al.*, 1994; Wikel, *et al.*, 1996). In all cases the sources of immunosuppression are salivary products transmitted to the host during feeding. The biochemical nature of active components varies between parasite and also between their chosen host and is, in some cases, still unidentified (Cross *et al.*, 1993; Wikel, *et al.*, 1994; Wikel, *et al.*, 1996).

Ribeiro *et al.* (1986) and Ribeiro (1987) report the inhibition of the alternative complement pathway and anaphylatoxins by *Ixodes dammini* in human, rat, mouse, hamster and guinea pig hosts. They identified a 49KDa protein present in salivary extracts that blocks the deposition of the C3b complement protein on the activating surface which subsequently prevents the complement cascade operating and the development of the C5b-C9 membrane attack complex.

The same extracts also blocked C3a and C5a release and in doing so prevented anaphylatoxin activity which is responsible for histamine release, increased blood vessel permeability and complement mediated chemotaxis by phagocytosis (Goldstein, 1988).

In addition to the action against host non-specific immune functioning, tick salivary gland extracts have been shown to affect cellular and humoral specific immunity. Wikel (1985) showed that guinea pigs infested with *Dermacentor andersoni* did not produce antibodies to the tick but produced antibodies to sheep red blood cells (SRBC) administered during the infection. However whilst antibody titres to SRBCs were 41% lower than in uninfected animals the titre was thought to offer sufficient protection to a subsequent challenge by the same antigen. It would seem then that the degree of immunosuppression is not sufficient to impair feeding by the parasite, but is adequate to protect the host from its environment obviously requiring some level of immunocompetence. The effect on specific immunity has been investigated primarily by monitoring *in vitro* responses of lymphocyte populations, responsible for antibody proliferation, to salivary gland extracts. *In vitro*, lymphocytes can be stimulated to proliferate by the application of concanavalin A (Con A). Con A stimulated rabbit lymphocytes were significantly depressed when collected during an infestation of *Ixodes ricinus* which returned to normal levels after the infection had ended (Schorderet & Brossard, 1994). Similarly the proliferative responses of stimulated T-lymphocytes from *Bos taurus* were reduced by 47% when cows were infected with *D. andersoni* (Wikel & Osbourne, 1982) and 44-49% when infected with *Boophilus microplus* (Inokuma *et al.*, 1993). Subsequently this suppression was attributed to Prostaglandin E_2 (Inokuma *et al.*, 1994) which had long been suspected as having immunosuppressive effects. However, these authors also showed that the negative effects of standard PGE_2 on T-lymphocyte proliferation *in vitro* was not as significant as that caused by whole salivary extract, indicating that the role of saliva proteins cannot be ruled out (Bergamn *et al.*., 1995). In addition to the effects on proliferative responses of lymphocytes, salivary extracts have been assayed for their effects on cytokine production by immune cells. Ramachandra and Wikel (1992) demonstrated that *D. andersoni* significantly suppressed Interleukin-1 (IL-1) and Tumour Necrosis Factor (TNF) production by murine macrophages *in vitro*. However, the mode of interference was different for both cytokines indicating the presence of multiple immunosuppressive factors. The most intense suppression of TNF production occurred using extracts harvested from ticks during the first few days of feeding. Following tick engorgement and removal from the host the suppressive effects of the salivary extracts against TNF reduced which suggests that the production of the suppressive component is stimulated by the feeding process and that it is not stored in an active form within the salivary glands. Ramachandra and Wikel (1995) also report the reduction in the rate of production of

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bovine macrophages during *D. andersoni* infection. Such modulation of immune cell functioning could impair both antibody and cell mediated immunity directly as well as interfering with the communication between the two interactive divisions of the immune system. Suppressed levels of IL-1 would impair T-lymphocyte activation; neutrophil mobilisation; cellular chemotaxis; IL-2 production; and Ig synthesis. Reduced TNF production would impair expression of MHC molecules; anti-viral responses and activation of granulocytes.

Vaccination against ticks such as *B. microplus* employs 'hidden' gut antigens not normally exposed to the host but which are immunogenic following inoculation, and elicit antibody production that act within the parasite gut during feeding (Willadsen, 1987; Wikel, 1988; Willadsen, *et al.*, 1989; Willadsen and McKenna, 1991). However, other strategies are also being explored by the same authors who hope to exploit the immunosuppressive mechanisms of the parasite to produce an 'anti-immunosuppresent' vaccine that will enhance host defences against tick feeding and against the transmission of tick-borne pathogens.

RESEARCH AIMS AND OBJECTIVES

The aims and objectives of the present research were to:

Investigate the immunological effects on Atlantic salmon of infection with *L. salmonis*. In particular the effects on macrophages and cellular immunity in an attempt to correlate these to the observations of reduced inflammatory responses at the site of parasite attachment reported by Johnson and Albright (1991). This work is presented in chapter 3.

Develop an *in vitro* cell culture system for the propagation of *L. salmonis* larvae to allow extended maintenance and a means of studying the parasite within a biologically controlled environment. Particular emphasis was placed upon collection of products secreted or excreted by the larvae to allow investigation of their effects on macrophage immune functioning. These areas of the study are presented in chapters 4, 5 and 6.

Identify the biochemical properties of louse products secreted into the culture system and to determine the effects of their manipulation on their ability to modulate macrophage immune functioning. This work is presented in chapter 7.

MATERIALS AND METHODS

2.1 EXPERIMENTAL ANIMALS

2.1.1 Infectivity Studies

Atlantic salmon, mean weight 250g were reared and maintained at the Experimental Fish Production Unit (EFPU), Aultbea in $1m^3$ tanks with flow-through seawater supply at ambient temperature. Rainbow trout, mean weight 280g, were reared and maintained at the same facility in $2m^3$ tanks with flow-through fresh water supply until 12 weeks prior to start of study when fish were transferred to $1m^3$ tanks with ambient seawater supply. During all experiments the fish were maintained on Trouw mainstream commercial salmon diets fed at 2% bodyweight per day.

2.1.2 Primary Cell Culture and *in vitro* Cellular Assays

Atlantic salmon, mean weight 1000g were reared at the EFPU, Aultbea and transferred to the Marine Laboratory Aquarium, Aberdeen. Fish were maintained in $1m^3$ tanks with flow-through seawater supply at ambient temperature.

2.1.3 Collection and Maintenance of Sea Lice

Ovigerous adult female sea lice were collected from farmed Atlantic salmon during harvesting on the west coast of Scotland. Lice were removed with forceps and placed in clean seawater from the site in plastic containers, packed with ice and transported back to the laboratory. At the laboratory, eggstrings were removed with forceps and placed in 5l beakers containing clean seawater, 33±2‰ at 10°C. The beakers were covered and incubated at 10°C with vigorous aeration. For the purposes of culture experiments, the progeny from batches of 10 female lice were maintained in separate beakers. Approximately 50% of the seawater volume was exchanged daily using filtration apparatus connected to a peristaltic pump. For challenge

experiments, approximately 50 eggstrings were incubated in 5l beakers under the same maintenance conditions.

THE COMPARATIVE IMMUNOLOGICAL EFFECTS OF *L. SALMONIS* INFECTION OF ATLANTIC SALMON AND RAINBOW TROUT

2.2 EXPERIMENTAL DESIGN

Four replicate $1m^3$ tanks each containing either 80 Atlantic salmon (mean weight 250g); 80 rainbow trout (280g); or 40 salmon with 40 trout were maintained at the Experimental Fish Production Unit, Aultbea (EFPU) as described in section 2.1.1. Duplicate tanks from each of the 3 groups were experimentally infected with *L. salmonis* copepodids, the remaining 2 tanks of each group were not infected and acted as controls.

Prior to challenge 4 fish from each tank were euthanised by anaesthetic overdose and blood collected as described in section 2.3. Samples of skin and dermal tissue were collected and stored in 8% neutral buffered formalin.

2.2.1 Experimental Infection

Tank volumes were reduced to $0.5m^3$, the outflow was covered by 40μm nylon mesh, the water flow was stopped and approximately 80 copepodid larvae per fish were added to each tank. Aeration was introduced after 30 minutes and a low flow rate initiated after 2 hours. After 6 hours the water flow was returned to the normal rate and the nylon mesh removed from the outflow the next day.

2.2.2 Sampling Regime

Fish from each tank were sampled on days 3,6,12,18,25 and 35 post infection. Ten fish from each tank were removed and anaesthetised using MS222 at 13mg/ml. Blood samples were withdrawn from the caudal sinus of all fish (section 2.3) and the total number and the developmental stage of *L. salmonis* recorded. Blood samples were used in the measurement of cellular composition (section 2.3), serum lysozyme activity (section 2.6), serum protein

concentration (section 2.5) and measurement of agglutinating antibody titres (section 2.7). The head kidney was also removed from three fish per tank and the tissue homogenised (section 2.8), before being stored on ice and transported to the laboratory for further processing and used in measurement of phagocytosis (section 2.9), respiratory burst (section 2.10) and chemotaxis (2.11). The remaining 7 fish were returned to the challenge tanks. The poor health of all challenged populations by day 35 of the experiment forced the premature end of this trial and did not permit the collection of blood and serum for use in serological assays, but head kidney was still sampled for the macrophage assays.

2.3 MEASUREMENT OF CELLULAR COMPONENTS OF PERIPHERAL BLOOD

Fish were euthanised by percussive stunning and peripheral blood sampled from the caudal vein using 4ml vacuettes and 26G needles (Griener), and used to measure packed cell volume and to determine the cellular composition before the collection of serum for subsequent serological assays (section 2.4).

2.3.1 Packed Cell Volume

Blood was collected from vacuettes into heparinised capillary tubes (Hawksley & Sons Ltd, UK). Duplicate samples were collected for each fish. Tubes were sealed at one end with Cristaseal (Hawksley & Sons Ltd, UK) and centrifuged at 15000g for 10 min in an haematocrit centrifuge (Heraeus). The packed cell volume was determined using a micro-haematocrit reader (Hawksley & Sons Ltd, UK) and expressed as percentage of the total blood volume.

2.3.2 Differential Cell Counts

A drop of blood was smeared across the surface of a glass microscope slide using the edge of a second slide and allowed to air dry. Duplicate slides were made from each fish sampled. Dried slides were fixed by immersion in 100% methanol and stained using HaemaGurr (BDH). Slides were viewed x100 and 200 cells per slide counted in random fields and the number of leucocytes recorded. In addition, 100µl of whole blood was diluted 1:10 in formal saline and transported back to the laboratory. It was further diluted to a final dilution of 1:1000 by the addition of 0.01M PBS pH7.4 and total cell counts made using a haemocytometer following the

technique described in section 2.14. The percentage of leucocytes recorded from blood smears was applied to the total cell counts to determine the composition of the samples in terms of numbers of white and red cells.

2.4 SERUM COLLECTION

Peripheral blood was collected by the method described above. Blood was allowed to clot at room temperature before centrifugation at 600g for 10 min. Duplicate 100µl aliquots of serum were made into 500µl micro-centrifuge tubes which were stored on dry ice for transport back to the laboratory for final storage at -80°C.

2.5 PROTEIN ESTIMATION

The protein content of culture supernatants and fish serum was measured using commercially available reagents, following the manufacturers instructions (Pierce, BCA Protein Assay Kit).

Briefly, 200µl of protein reagent was added to BSA standards, distilled water blanks and duplicate serial dilutions of serum/supernatant made in distilled water, in a 96 well flat bottom microtitre plate (Nunc). The plate was incubated for 30 min at 37°C and the absorbance measured at 540nm on a Dynex Dias plate reader using Dynex Revelation V3.1 analysis software . The protein concentration was calculated from a standard curve for each dilution and the mean value of those dilutions taken for each sample and expressed as protein mg/ml.

2.6 MEASUREMENT OF SERUM LYSOZYME ACTIVITY

This method is a modification of the turbidimetric lysozyme assay described by Ellis (1990). Twenty-five microlitres of serum, and 25µl of 0.01M PBS pH7.4 as a negative control, were dispensed into duplicate wells of a 96 well microtitre plate. A 0.2mg/ml *Micrococcus lysodeikticus* suspension in PBS pH6.4 was then added to all wells at 175µl per well. The absorbance was read at 540nm every minute for 20 min against a blank composed of 200µl PBS.

One unit of lysozyme is defined as a decrease in absorbance of 0.001/min. The total lysozyme content of each sample is determined by:

$$\text{Lysozyme Activity} = \frac{\text{The total decrease in O.D over period}}{\text{Length of period (min)}}$$

2.7 AGGLUTINATION ASSAYS

Sera were serially diluted (1:2 to 1:1024) in 0.01M PBS pH7.4 to a final volume of 50µl in 96 well V-form microtitre plates. Either 50µl of latex bead suspension (for louse homogenate agglutination) or 50µl of *Aeromonas salmonicida* suspension (for bacterial agglutination) was added to all wells and the plate incubated overnight at 4°C. Titres were recorded as the reciprocal of the last dilution in which visible agglutination occurs. Sera from each day of the trial, except from day 35, were assayed. Samples were not collected post infection as originally planned due to the poor health of the challenged population. Assays were performed in duplicate, and wells containing only PBS acted as negative controls.

2.7.1 Preparation of Louse Homogenate Coated Latex Beads

Ten pre-adult sea lice were macerated and then further dissociated by vortexing with 10ml of 10% v/v sterile sand in 0.01M PBS PH7.4. The supernatant was filter sterilised (0.22µm, Millipore) and 10µl 0.5g/ml Phenylmethanesulfonyl fluoride (PMSF, Sigma) added. The protein content of the solution was calculated (section 2.5) and aliquots stored at -20°C.

One ml of a 10% suspension of 3µm latex beads (Sigma) was washed 3 times in 0.05M carbonate-bicarbonate buffer pH 9.6. The suspension was diluted 1:10 with buffer and louse homogenate added at 20µg/ml. Following mixing overnight at 22°C the latex beads were centrifuged at 600g for 5 min and the pellet resuspended in 5% skimmed milk powder (Sigma). The suspension was incubated for a further 4 hours at 22°C and then washed 3 times with 0.01M PBS pH7.4 by centrifugation at 600g.

2.7.2 Preparation of Bacterial Suspension

Aeromonas salmonicida (MT004) was resuscitated from storage and grown on Tryptone Soya Agar (Oxoid) for 48 hours at 22°C. Several colonies were removed and used to inoculate 200ml of Tryptone Soya Broth (Oxoid) and grown for 48 hours at 22°C, 140rpm. The bacteria were inactivated by the addition of formalin to give a final concentration of 0.5% v/v and the cell suspension incubated for a further 24 hours. The bacteria were washed 3 times with 0.01M PBS pH7.4 and the concentration adjusted to $1x10^9$ cells/ml (Optical density 1.0 at A540nm).

2.8 MACROPHAGE ISOLATION

Head kidney macrophages were isolated according to the method of Secombes (1990). Fish were euthanised by anaesthetic overdose and exanguinated by severing of gill arches bilaterally. The anterior kidney was dissected out within an aseptic environment and dissaggregated through 1mm steel mesh into ice-cold L-15 (meshing). The suspension was further dissaggregated through mesh of 100μm and finally 50μm pore size before being layered onto a 34%-51% discontinuous Percoll® density gradient (Pharmacia). The gradient was centrifuged at 400g for 40 min at 4°C with no brake. Cells were harvested from the interface of the 33% and 51% Percoll layers and washed twice in L-15 (washing). Viable cell counts were performed using a haemocytometer and the trypan blue exclusion method as described by Phillips (1973) (section 2.14). The cell suspension was adjusted to the required concentration in L-15 (washing).

L-15 (meshing)

Leibovitz medium (L-15)
0.1% heparin
2% foetal calf serum
1% Pen/Strep Solution
(5000IU/ml Penicillin and 5mg/ml Streptomycin, Sigma)

L-15 (washing)

L-15 medium
0.1% heparin
0.1% foetal calf serum
1% Pen/Strep Solution

2.9 PHAGOCYTOSIS ASSAY

The phagocytic activity of head kidney macrophages was evaluated using the microscope counting technique described by Secombes (1990). One hundred microlitres of $1x10^7$ macrophages/ml was added to each well of 16 well chamber slides (Nunc). Cells were allowed to adhere overnight at 15°C and non-adherent cells removed by washing with 0.01M PBS

pH7.4. One hundred microlitres of a suspension of yeast (*Saccharomyces cerevisae*) (Sigma), heat killed and at a concentration of $1x10^7$ cells/ml in PBS was added to each well and incubated in a humid box at 22°C for 60 min. Following incubation the cells were washed 3 times in PBS and then fixed with 70% methanol. The chambers were removed and the slides stained with differential stain (HaemaGurr, BDH). Slides were viewed at x100 oil immersion and 200 cells examined. The number of cells that had phagocytosed was noted and also the number of yeast cells that these macrophages had engulfed. This method allows the calculation of both phagocytic activity and the phagocytic index:

2.10 MEASUREMENT OF INTRACELLULAR RESPIRATORY BURST

This assay measures intracellular superoxide anion production by macrophages through the reduction of nitro-blue tetrazolium (NBT, Sigma), following the method of Secombes (1990). One hundred microlitres of $1x10^7$/ml head kidney macrophages were added to wells of 96 well microtitre plates and allowed to adhere overnight at 15°C. Non-adherent cells were removed by washing with 0.01M PBS pH7.4. One hundred microlitres of either 1mg/ml NBT in HBSS, 1mg/ml NBT + 1µg/ml PMA, or 1mg/ml NBT + 1µg/ml PMA + 300µg/ml superoxide dismutase (SOD) was added to triplicate wells of each macrophage treatment group and incubated at 15°C for 60 min (determined following optimisation). The reaction was stopped by the removal of reagents and the addition of 100% methanol. Monolayers were washed 3 times with 70% methanol and then 120µl of dimethyl sulphoxide (DMSO) and 140µl of 2M potassium hydroxide (KOH) added to release intracellular formazan.

$$\text{Phagocytic activity (\%):} \quad \frac{\text{No. cells phagocytosing}}{\text{Total No. cells counted (200)}} \times \frac{100}{1}$$

$$\text{Phagocytic index :} \quad \frac{\text{Total No. yeast phagocytosed}}{\text{No. cells phagocytosing}}$$

The absorbance of the released dye was read at 630nm against a blank of DMSO/KOH. Additional wells were maintained without the addition of the substrates to act as cell counting wells. The cell number was estimated following the dye exclusion method in section 2.14, and superoxide anion production then expressed as $OD/10^5$ cells.

2.11 MACROPHAGE MIGRATION ASSAY

Chemotaxis by salmon head kidney macrophages was investigated using 96 well microchemotaxis chambers (ChemoTx®, NeuroProbe Inc., USA) following manufacturers instructions but after optimisation for use with salmon material. Head kidney cells were isolated as described in section 2.8 and foetal calf serum, a potential chemoattractant, removed by repeated washing in serum free L-15 medium. Thirty microlitres of 5% salmon serum in 0.01M PBS pH7.4, and PBS alone as the negative control, were placed in the lower chambers. A 5μm polycarbonate filter was then lowered onto the lower chambers and 50μl of $1x10^6$/ml macrophages added to the inlaid wells. The apparatus was covered and incubated for 60 min in a humid box at 15°C. Samples were assayed in triplicate wells against both serum and PBS. After incubation the filter was removed and the upper surface washed in a stream of PBS and the lower surface stained using a differential staining kit (HaemaGurr, BDH). The number of migrated cells on the lower surface was then assessed microscopically in 4 random fields and expressed as number of cells per field of view. The net movement of cells was determined by subtracting the number of cells migrating towards the control from the number migrating towards the serum.

THE DEVELOPMENT OF AN ORGANOTYPIC FISH SKIN MODEL

2.12 ROUTINE CULTURE OF ESTABLISHED CELL LINES

Four established cell lines were maintained for the duration of this project; Atlantic Salmon Epithelium (ASE-W) and Atlantic Salmon Fibroblast (AS-6) courtesy of The Centre for Environment, Fisheries and Aquacultural Science, Weymouth, UK; Chinook Salmon Embryo (CHSE-214), and Rainbow Trout Gonad (RTG-2) supplied by Fisheries Research Services, Marine Laboratory, Aberdeen, UK.

2.12.1 ASE-W Cell Line

The ASE-W cells are an epithelial cell line derived from ectoderm of Atlantic salmon embryo. They were routinely cultured using L-15 (Life Technologies Ltd.) + 10% FCS + 1% (v/v) penicillin - streptomycin solution (5000 IU/ml - 5mg/ml, Sigma). Cells were maintained at 15°C and passaged 1 flask to 3 approximately every 4 weeks.

2.12.2 AS-6 Cell Line

The AS-6 cells are a fibroblast line derived from normal heart, liver, kidney and spleen tissue of Atlantic salmon. They were maintained at 15°C in L-15 supplemented with 10% FCS + 1% (v/v) penicillin - streptomycin solution + 30mM NaCl and passaged 1:3 approximately every 4 weeks.

2.12.3 CHSE-214 Cell Line

The CHSE cells are an epithelial line derived from non-specific embryonic tissue of Chinook salmon. They were routinely cultured using MEM (Life Technologies Ltd.) + 10% FCS + 1% (v/v) penicillin - streptomycin solution, maintained at 15°C , and were passaged 1:3 approximately every 4 weeks.

2.12.4 RTG-2 Cell Line

The RTG cells are a fibroblastic line derived from the gonad of rainbow trout. They were cultured using MEM supplemented with 10% FCS + 1% (v/v) penicillin - streptomycin solution, maintained at 15°C , and were passaged 1:3 approximately every 4 weeks.

2.13 Routine Passage of Cell Lines

Medium from confluent cultures was removed by aspiration with a pipette and the cell sheet washed with Dulbecco's PBS (Life Technologies.). The monolayer was dissociated by the addition of 0.25% trypsin – 1mM EDTA solution (Life Technologies) at a volume sufficient to cover the culture surface. An equal volume of culture media with serum was then added to neutralise the trypsin and the suspension repeatedly passed through a pipette in order to break up large aggregations of cells. Aliquots of cell suspension were added to new culture flasks containing appropriate volumes of medium to obtain the required passage ratio.

2.14 Calculation of Cell Culture Population Growth

(Dye exclusion assay – Phillips, 1973)

The growth and survival of cultured cells was determined at 24 hour intervals. At each time point cells from duplicate culture wells of each treatment group were suspended by the removal of culture media and the addition of trypsin-EDTA solution (Life Technologies) (at half the removed volume). Following lifting of cells from the culture surface, a volume of normal culture medium, equal to the enzyme volume, was added and large aggregations of cells

dissociated by repeated pipetting. The number of viable cells was calculated by the dye exclusion method of Phillips (1973). The test was performed as follows:

Typically, HBSS was added to cell suspensions to a final dilution of x10. An equal volume of 0.4% (w/v) trypan blue solution was then added and the suspension allowed to stand for 1 minute. A sample was then added to a prepared haemocytometer (Sigma) and the number of unstained cells counted in 5 squares of both chambers. A second sample was withdrawn and the count repeated. The number of cells per ml was determined by:

Cell Number per ml = Average count per square X dilution factor X 10^4

2.15 Calculation of Cell Population in Organotypic Systems

Cell population growth and survival was calculated at 24 hour intervals. Duplicate wells of each treatment group were assayed. The structure of each well was disrupted by maceration with a sterile loop. Five hundred microlitres of trypsin-EDTA solution was added to each well and the content further homogenised with a loop and then by repeated pipetting. The suspension was centrifuged at 200g with no brake in order to pellet only the larger pieces of collagen. The supernatant was withdrawn and the enzyme neutralised by the addition of normal culture media. The approximate contact time of cells and enzyme was 7 minutes. A sample from this suspension was used in haemocytometer counting as described in section 2.14.

In the optimisation of this method the pelleted collagen was examined for non-dissociated cells and it was determined that the above method was consistently effective at liberating >96% of the total cell population

2.16 Survival of Larval *L. salmonis* in Sub-optimum Conditions

The physiological optimum conditions for *L. salmonis* are within a seawater environment, however, the physiology of cultured animal cells requires an aqueous environment with a much lower salt balance containing a lower proportion of sodium chloride. The differences can be seen in terms of osmolarity where seawater is approximately 1000 $mOsmol.kg^{-1}$ and standard serum containing media is around 350 $mOsmol.kg^{-1}$.

To investigate the tolerance of *L. salmonis* larvae to lowered osmolarity 10 copepodids, previously maintained following the methods in section 2.1.3, were added to each well of 12 well tissue culture plates in 2ml of sterile seawater. Following 2 hours of acclimatisation the seawater was replaced by either seawater diluted by the addition of distilled water to osmolarities from 300 mOsmol.kg^{-1} to 1000 mOsmol.kg^{-1} at 100 mOsmol.kg^{-1} increments, or unsupplemented Leibovitz media (L-15) (300 mOsmol.kg^{-1}), L-15 supplemented with 10% FCS (350 mOsmol.kg^{-1}), L-15 supplemented with 1% FCS (320 mOsmol.kg^{-1}) or unsupplemented Minimum Essential Media (MEM) (300 mOsmol.kg^{-1}), MEM plus 10% FCS (350 mOsmol.kg^{-1}) or MEM supplemented with 1% FCS (320 mOsmol.kg^{-1}). Each solution was applied to duplicate plates and the plates maintained in humid boxes at 10°C. Each well was observed daily and the number of surviving lice noted. For the purposes of this experiment mortality was defined as occurring when an individual was no longer able to maintain its position in the water column and where all appendage and gut movements had stopped.

2.17 Osmotic Adaptation of Fish Cell Lines

The osmolarity of the culture medium used to maintain the established cell lines in this study (section 2.12) is approximately 300-350 mOsmol Kg^{-1}. Hyperosmotic media (Osmedia) was prepared either by adjusting the volume of liquid added to powered media formulations, or by the addition of magnesium chloride and sodium sulphate, or a combination of mannitol, sorbitol and xylose (Sigma) which act as osmopotentiators. The osmolarity of the media was determined using a Wecor 5500C Vapour Pressure Osmometer.

Osmolarity (mOsmol Kg^{-1})	Salt Solution (v/v normal media)	Sugar Solution (v/v normal media)	Salt/sugar Solution (v/v normal media)
500	2%	6%	5%
600	4%	9%	7%
700	6%	14%	9%
800	8%	18%	12%
900	11%	20%	14%

Salt solution: 50% v/v 5M MgCl, 50% v/v 2.5M Na2SO4 pH7.4
Sugar solution: 33.3% v/v 1M sorbitol, 33.3% v/v 1M mannitol, 33.3% v/v 1M xylose, pH7.4
Salt/sugar solution: 50%v/v salt solution, 50% v/v sugar solution, pH7.4

Medium was removed from monolayer cultures when cells were approximately 50% confluent (3-4 days post passage) and replaced by media at 500 mOsm Kg^{-1} (osmedia 400). Cells were maintained until confluency and passaged using standard methods (section 2.13). Cell viability and proliferation rates were monitored using the trypan blue exclusion assay (section 2.14) on duplicate cultures sampled every 2 days. Cultures showing good viability were incubated with media of increased osmolarity using the method described above, but only following a minimum of 3 passages in each osmedium. Sample cultures in each osmedium were maintained without further osmolarity increases to observe their persistence following adaptation.

2.18 Measurement of Osmolarity of Culture Media and Supernatants

Osmolarity was measured using a Wescor 5500C Vapour Pressure Osmometer following the instructions given by the manufacturer. Briefly, 10µl of filtered supernatant, medium or seawater is applied to a disc of filter paper that is then inserted into the calibrated osmometer and the osmolarity measured and displayed in milliosmoles per kilogram.

2.19 Extraction of Type I Collagen from Rats Tails

This method is a modification of that presented by Elsdale & Bard (1972). The tails of 20 Sprague-Dawley rats were obtained from the University of Aberdeen Medical School and stored at -20°C until required. Tails were thawed at room temperature for 4 hours. All subsequent procedures were performed in a sterile environment. Tails were individually immersed in 100% ethanol and then broken into 3-4cm lengths using sterilised pliers. Gentle pulling apart of the broken sections exposed the tendons which were cut and placed into ice-cold 0.5M acetic acid (one litre of acetic acid is required for primary extraction of collagen from approximately 25 tails). The mixture was then incubated at 4°C with continual stirring for 24 hours. The solution was passed through a coarse sieve to remove gross insoluble material which was added to one litre acetic acid to allow extraction to continue for an additional 24 hours. The tropocollagen solution produced from the primary extraction was stored at 4°C and was subsequently mixed with the solution from the secondary extraction which had been first passed through a sieve. The solution was then dialysed against 8 changes of distilled water at 4°C over a 48 hour period and then centrifuged at 4000rpm for 4 hours at 4°C. The supernatant is retained and its collagen content determined by measuring the absorbance at 230nm against a standard curve produced by absorbance measurements of serially diluted type I murine collagen

(Sigma). The solution was diluted to 3mg/ml in distilled water and stored at 4°C, the extracted tropocollagen is nearly always sterile and the addition of antibiotics is not required.

2.20 Formation of Collagen Gels

The exact method for collagen polymerisation is particular to each batch of tropocollagen prepared. The production of native fibre collagen gels requires a rapid increase in the pH and ionic strength of the tropocollagen solution which was achieved by the addition of x10 Hanks Balanced Salt Solution (HBSS) and 7.5% sodium bicarbonate. Typically, 15%v/v x10 HBSS and 2% v/v 7.5% sodium bicarbonate solution were added to a 3mg/ml tropocollagen solution. The collagen was used immediately since polymerisation prevented accurate dispensing within 3-4 min at room temperature.

2.21 The Use of Collagen Substrate For The Culture of Fish Cells

One hundred microlitres of polymerising collagen (section 2.20) was added to each well of a 24 well cell culture plate (Nunc). The collagen was spread evenly across the plate surface using a sterile plastic loop and allowed to polymerise within a sterile environment to form a basement layer. Eight replicate plates were prepared for each fish cell type used (section 2.12). Cells were harvested from stock flasks using trypsin-EDTA as described in section 2.13 and the concentration adjusted to 2 x 10^5 cells/ml in normal culture media. Five hundred microlitres of this suspension was added to the collagen substrate in each well and cells cultured under normal conditions as described in section 2.12. Control plates were also made of each cell type which were cultured without the collagen basement. At 24 hour intervals the cell density of duplicate culture wells from each group was determined following the method laid out in section 2.14.

2.22 The Use of Collagen Substrate and a Seawater Medium For The Culture of Fish Cells

This experiment was conducted as described in section 2.21 except that in this case cells were cultured until peak density was reached, approximately 10 days post passage. At this time the acute adaptive responses of cells to survive in seawater media were tested by the replacement of normal culture medium by a 25% seawater medium (500 mOsmol.Kg^{-1}) (standard powdered

medium prepared using 75% distilled water and 25% filtered seawater (33 ± 2 ‰)). Half of the culture plates (4) were maintained using this medium for the duration of the experiment. The remaining 4 plates were maintained in the 25% seawater medium for 7 days after which the medium was replaced by a 50% seawater media (800 mOsmol.Kg^{-1}). The cell density was determined at 24 hour intervals as previously described (section 2.14). Control plates were maintained in normal culture medium without the addition of seawater.

2.23 The Use of a Collagen 'Sandwich' Configuration For The Culture of Fish Cells

This configuration included the addition of a secondary layer of collagen to the surface of cultured cells used in sections 2.21 and 2.22, and can be seen in diagrammatic form in figure 2.1a. At peak cell density, approximately day 10 post passage, the culture media was removed and the cell surface washed in 0.01M PBS, pH 7.4. One hundred microlitres of collagen was added to the surface of the cell monolayer and allowed to polymerise. The structure was maintained without medium for 2 days after which either normal medium or sterile seawater (33 ± 2 ‰) (~1000 mOsmol.Kg^{-1}) was added. Duplicate wells of each plate were sampled daily and cell number calculated according to section 2.15. This experiment was performed using all cell types (section 2.12) and 8 replicate plates. Controls consisted of cells maintained by normal culture media with a collagen substrate (as per section 2.21) until day 12, at which time medium was replaced by sterile seawater.

2.24 The Use of a Bilayered Collagen 'Sandwich' For The Culture of Fish Cells

This configuration adds both an additional cell layer and collagen layer to the previous 'sandwich' configuration (section 2.23), but also includes a modified collagen layer. The structure is represented diagrammatically in Figure 2.1b. Only Atlantic salmon epithelial cells (ASE-W) and fibroblasts (AS-6) were used in this experiment.

One hundred microlitres of collagen was added to wells of 8 replicate, 24 well tissue culture plates and allowed to polymerise. The surface was washed in HBSS and 0.5ml of a $2x10^5$ cells/ml suspension of cultured cells added. Cells were allowed to proliferate for 7 days by which time they were confluent, but not at peak density. Medium was removed and 100µl of a

collagen-cell suspension was layered onto the surface of the cell monolayer. The collagen-cell suspension was formed by the addition of a $1x10^8$ cells/ml cell suspension at 10% v/v of polymerising collagen solution (section 2.20) to give an approximate cell density of $1x10^6$ cells/ml. This layer was allowed to polymerise overnight before the addition of a further collagen layer (100µl). Following polymerisation the structure was maintained without medium for 2 days. After this time the structures were incubated with 1.3ml normal culture medium. At day 24 post passage, when approximate maximum cell density was achieved, medium was removed from all plates and replaced by fresh normal culture medium in half of the plates, and by sterile seawater (32 ± 2 ‰) in the remaining plates. Cell density was calculated at 24 hour intervals (section 2.15). Controls consisted of cells cultured within a collagen sandwich configuration (section 2.23) using normal medium.

2.25 The Culture of Fish Cells Within a Multi-layered Salmon Skin Equivalent

Previous cell-collagen constructs used in this project incorporated only a single cell type. The creation of an Atlantic Salmon Skin Equivalent (ASSE) was the next logical step in the development process of *in vitro* culture systems used in this study. The ASSE uses an established salmon fibroblast cell line (AS-6) and primary cultures of Atlantic salmon epithelial cells within a collagen framework comparable to those used in the formation of mammalian skin equivalents. A diagrammatic representation of the structure can be seen in Figure 2.1c. Experiments using ASSE followed the same pattern as previous studies in that 8 replicate 24 well plates were prepared and duplicate wells assayed at 24 hour intervals and cell density determined according to the methods in section 2.15.

The absence of a fibroblastic chinook salmon cell line and an epithelial rainbow trout cell line meant that CHSE and RTG skin equivalents used in this project were composed of only single cell types but incorporated within the same multi-layered structure as the ASSE.

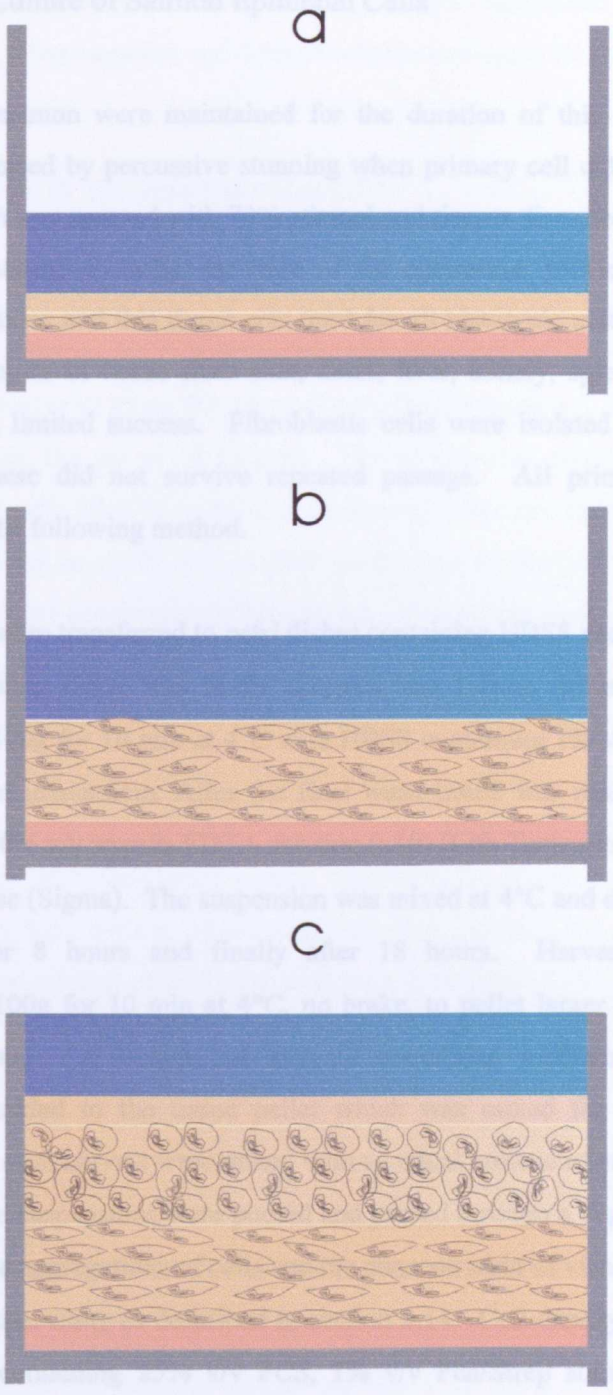

Figure 2.1

Diagrammatic representation of tissue culture substrate development.

a) Collagen Sandwich configuration (section 2.23) – single layer of single cell type with basement and surface collagen layers

b) Bilayered Collagen Sandwich configuration (section 2.24) – single layer and multi-layer of single cell type in collagen matrix

c) Multi-layered Salmon Skin Equivalent (ASSE) (section 2.25 – multiple layers of fibroblast and epithelial cells in collagen matrix

2.25.1 Primary Culture of Salmon Epithelial Cells

Sibling Atlantic salmon were maintained for the duration of this study (section 2.1.2) and individuals euthanised by percussive stunning when primary cell cultures were required. The exterior of the fish was sprayed with 70% ethanol and tissues dissected in a sterile environment. The opaque membrane covering the edge of the operculum provided the longest surviving epithelial cell cultures and this tissue was used for all histotypic cultures performed as part of this project. Samples of tissue from skin, heart, liver, kidney, spleen and gonads were also cultured but with limited success. Fibroblastic cells were isolated from skin and opercular membrane but these did not survive repeated passage. All primary cell isolations were performed using the following method.

Dissected organs were transferred to petri dishes containing HBSS and any fat or necrotic tissue removed. Remaining tissue was finely chopped into 1-2mm cubes and washed 3 times by centrifugation at 100g for 10 min at 4°C with HBSS containing 1% v/v Pen/Strep solution and 0.2% v/v 50mg/ml gentamicin. After the final wash tissue was resuspended in 10 times the pellet volume of 10% v/v trypsin-EDTA solution (x10) (Life Technologies) in HBSS containing 4mg/ml collagenase (Sigma). The suspension was mixed at 4°C and dissociated cells harvested every 60 min for 8 hours and finally after 18 hours. Harvesting was performed by centrifugation at 100g for 10 min at 4°C, no brake, to pellet larger pieces of tissue and the supernatant retained. A volume of enzyme containing medium equal to the removed supernatant was added to the tissue pellet which was mixed for a further 60 min. The supernatants from each harvest were mixed with an equal volume of standard L-15 medium and stored at 4°C. The suspensions were pooled and passed through a 50μm cell dissociation sieve to break apart large aggregations of cells and the concentration of viable cells determined by the trypan blue exclusion assay as described in section 2.14. Cell density was adjusted to $0.5x10^6$ cells/ml in L-15 containing 25% v/v FCS, 1% v/v Pen/Strep solution, 0.2% v/v 50mg/ml Gentamicin and 0.5mM calcium carbonate and 400μl dispensed into wells of 24 well culture plates (Nunc) and incubated at a range of temperatures. The optimum culture temperature was 22°C. Non-adherent cells were removed after 48 hours by gentle pipetting of the culture media. Cultures were observed daily and the medium changed after 5 days and thereafter weekly until the first passage at around week 3. Initial media changes involved the mixing of fresh medium with 50% v/v of the aspirated medium which was firstly filter sterilised (0.22μm, Millipore). Passage and cell maintenance followed standard methods as described in section 2.13, except that gentamicin was excluded following the first passage and the FCS content was reduced by

5% at this and each subsequent passage until cells were maintained in L-15 media containing 10% FCS, 1% Pen/Strep solution and 0.5mM calcium carbonate following passage 3.

2.25.2 Construction of skin equivalent (ASSE).

The construction method for ASSE is summarised in Figure 2.2. A basement layer of type I collagen gel approximately 3mm in depth was added to wells of 24 well culture plates (Nunc). Collagen was polymerised using the method described in section 2.20 to give a final collagen concentration within the gel of ~2.8mg/ml. The surface was seeded with fibroblasts at 0.5-1x10^6 cells/ml and cells allowed to adhere. Following adherence the medium was removed and the cell sheet washed in sterile 0.01M PBS pH7.4 and the surface then allowed to dry in a sterile environment for approximately 10 minutes. Fibroblasts were harvested from monolayer culture and cell density adjusted to 1x10^8/ml by centrifugation. The cell suspension was mixed with collagen at 10% v/v. $NaHCO_3$ and x10 HBSS are added to the cell/collagen suspension and 100µl layered onto the surface of the fibroblast monolayers where the mixture gels. An approximate cell density of 1x10^6/ml was achieved. Cultures were maintained in Leibovitz medium containing 10% foetal calf serum, 1% Penicillin-Streptomycin solution and 30mM sodium chloride for 8-12 days by which time the cells contracted the collagen to form a dermal equivalent. Primary cultures of epithelial cells were incorporated into collagen matrix using the same method and were then added to the surface of the dermal equivalent. Cultures were propagated in L-15 medium for a further 10-15 days. The construction was completed by the addition of an acellular layer of collagen which, when polymerised was maintained in x3 HBSS (700 mOsmol.Kg^{-1}) for 6 days. Mature ASSE was formed approximately 35 days following the start of the construction.

2.25.3 Histological Processing of Tissue Culture Configurations

Histological evaluation of ASSE structure and effects of subsequent louse incubation and biochemical supplementation were made using standard methods. Briefly, substrates were fixed *in situ* by the removal of culture media and addition of 10% buffered formal saline. After fixation, samples were removed and loaded into histological cassettes and dehydrated through a graded alcohol sequence before final embedding in paraffin wax. Five micron sections were cut

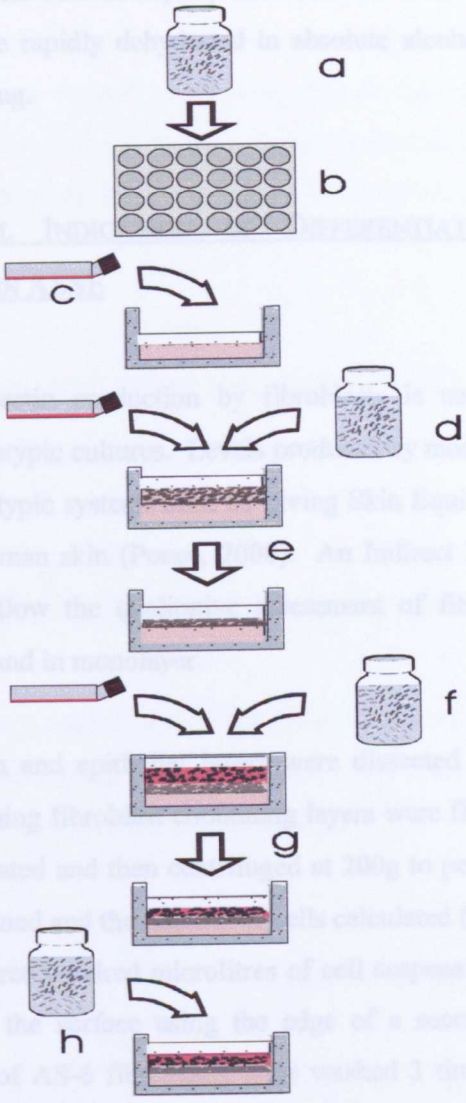

Figure 2.2. Diagram of the formation of Atlantic Salmon Skin Equivalent (ASSE)

a: Solution of type I collagen, acid-extracted from tails of rats. **b:** Basement layer of collagen is added to plastic culture surface and polymerised by addition of x10 HBSS and sodium hydrogen carbonate. **c:** Cultured fibroblasts (AS-6), derived from mixed tissue homogenate of Atlantic salmon, are added to collagen surface following polyermisation and cells allowed to adhere. **d:** Fibroblasts are mixed with collagen solution and added to culture well and allowed to gel. **e:** A dermal equivalent is formed by fibroblast contraction of the collagen matrix after 8-12 days culture at 22°C. **f:** Epithelial cells, derived from primary culture of Atlantic salmon opercular tissue are maintained until passage 8 and combined with collagen and seeded onto the surface of the dermal equivalent. **g:** The cultures are maintained in media for up to 15 days. **h:** A surface layer of acellular collagen is added to the 'epidermis' and maintained in x3 HBSS for approximately 6 days. The ASSE is mature and suitable for incubation with seawater and subsequent testing approximately 35 days from start of construction.

and stained with Harris Haematoxylin, differentiated in acid alcohol and counterstained with eosin. Sections were rapidly dehydrated in absolute alcohol and cleared using clearene and xylene before mounting.

2.26 Biochemical Indication of Differentiated Functioning of Cells Maintained Within ASSE

The level of fibronectin production by fibroblasts is used as an indicator of tissue-like functioning of organotypic cultures. Levels produced by monolayer cultures is much lower than those seen in organotypic systems such as Living Skin Equivalents where they are comparable to those found in human skin (Ponec, 2000). An Indirect Fluorescent Antibody Test (IFAT) was developed to allow the qualitative assessment of fibronectin production by fish cells cultured with ASSE and in monolayer.

The surface collagen and epithelial layers were dissected from mature ASSE (>35 days of culture). The remaining fibroblast containing layers were flooded with 0.01M PBS pH7.4 and mechanically dissociated and then centrifuged at 200g to pellet larger pieces of collagen. The supernatant was retained and the number of cells calculated (section 2.15) and adjusted to $1x10^6$ cells/ml in PBS. Three hundred microlitres of cell suspension was dropped onto a glass slide and smeared across the surface using the edge of a second slide and allowed to air dry. Monolayer cultures of AS-6 fibroblasts were washed 3 times in PBS and resuspended using sterile cell scrapers (Life Technologies). The cell number was adjusted to $1x10^6$ cells/ml and applied to glass slides as above.

Glass slides containing cells were blocked by the incubation with 6% skimmed milk solution for 30 minutes. Slides were removed, drained and incubated with a mouse anti-human fibronectin monoclonal antibody (Sigma) at a 1:1000 dilution with 1% skimmed milk solution for 1 hour. Slides were then washed twice in PBS containing 0.1% v/v Tween 20 (PBST) for 2 minutes and then incubated for 1 hour with an FITC conjugated goat anti-mouse IgG monoclonal antibody diluted 1:1000 in 1% skimmed milk solution. Slides were then blocked for 30 minutes in 6% skimmed milk solution, drained and incubated with Citifluor (Citiflour Inc, USA), an FITC stabilising agent, diluted 1:4 with PBST for 10 minutes before a further blocking stage. Slides were then counterstained with 0.01mg/ml propidium iodide in PBST for

2 minutes and then blocked with 6% skimmed milk solution for 30 minutes. Slides were allowed to drain before 2 drops of Citifluor were added to the surface prior to attachment of coverslip. Slides were viewed under x10, x20 & x40 objectives on an Olympus BX60 fluorescent microscope using an I3 fluorescent excitation filter (FITC broad band). Positive cells appeared red with green FITC staining in the cytoplasm, negative cells appear red but lack the green staining.

THE USE OF ATLANTIC SALMON SKIN EQUIVALENT FOR THE MAINTENANCE OF *L. SALMONIS* LARVAE *IN VITRO*

2.27 NORMAL *IN VITRO* CULTURE CYCLE

ASSE was cultured until approximately day 32 as described in section 2.24.2, in 12 wells of 24 well tissue culture plates (Nunc). Each culture cycle consisted of at least 4 replicate plates. Each plate contained 4 groups each with 6 replicate wells:

Name of group/supernatant	*Culture Conditions*	*Abbreviation*
Seawater	Seawater only - No ASSE, No Lice	SW
Louse Control	Lice & Seawater only - No ASSE	LC
Matrix Control	ASSE & Seawater only – No Lice	MC
Louse Culture Supernatant	ASSE, Lice & Seawater	LCS

All procedures were performed in a sterile environment. Following disinfection (section 2.27.2), 5 copepodids were added to each well of the LC and LCS groups in 1.3ml of seawater. The same volume of seawater alone was added to the remaining groups. Plate lids were added to culture plates which were incubated in humid boxes at 10°C. Plates were maintained in a 12 hour dark:12 hour light photoperiod and arranged randomly with respect to the position or the light source. The medium/supernatant from all wells was collected as described in section 2.27.3, and observations of copepodid survival in each well were made daily using the criteria defined in section 2.16. For the purposes of this project 4 replicate 'normal' culture cycles were

performed using 4 separate batches of copepodids collected between September 1998 and July 1999.

2.27.1 Disinfection of Copepodids

Newly hatched copepodids were harvested from the stock vessel using a 10ml serological pipette and transferred to a petri dish. Animals were examined using a binocular dissecting microscope at x20 magnification, and approximately 100 individuals transferred by plastic Pasteur pipette to 30ml universal tubes (Norlab) containing disinfection medium (sterile seawater + 1%v/v Pen/Strep solution + 1%v/v 50mg/ml gentamicin).

All subsequent procedures were performed in a sterile environment. Copepodids were incubated in 3 washes of disinfection medium, each wash lasting approximately 2 hours at 10°C. At each wash, 75% of the medium was removed using microtubing (Microflex) with a 40μm filtered end connected to a peristaltic pump operated at low flow rates to avoid suction damage to the lice. Following the third incubation period the disinfection media was replaced by 2 washes of sterile seawater, each of 10 min. Disinfected copepodids were immediately used in the culture system.

2.27.2 Collection of Culture Supernatants

One millilitre of culture medium (seawater) was removed daily from each well of 24 well culture plates for the duration of the culture cycle. Collection normally continued only to day 14, by which time copepodid mortality was normally > 80%. Corresponding media from each plate were pooled to give approximately 6ml of supernatant from each of the culture groups. Following centrifugation at 600g for 10 min to remove particulates, the culture supernatants were dialysed against 2 changes of 0.01M PBS pH7.4 at 4°C over a 3 hour period to reduce the osmolarity of the sample to approximately 350 $mOsmol.Kg^{-1}$. One millilitre aliquots of dialysed supernatants were made in cryotubes, labelled and stored in liquid nitrogen for future analysis.

Alternative methods of de-salting the supernatants such as microconcentration and gel-filtration (PD10, Amersham Pharmacia Biotech) were trialled but the dialysis method was the most appropriate given the sample volumes and the number of samples. However, smaller sample volumes up to 1ml were dialysed using 5000 Dalton molecular weight cut off Spin Biodialysers (Sialomed Inc, USA).

2.27.3 The Effect of Copepodid Culture on ASSE Structure and Cell Viability

During a normal copepodid culture cycle, one well containing ASSE and copepodids from duplicate plates was sampled at 24 hour intervals. These wells were viewed microscopically to allow observations of gross structural damage caused by louse behaviour and the number of viable cells remaining in the structure calculated.

Qualitative observations of the pattern and extent of damage in terms of the approximate area of cell loss in the surface layers and effects on deeper layers were recorded. Following these, the ASSE was deconstructed using the methods described in section 2.15 and the number of remaining viable cells determined

2.28 PERFORMANCE OF COPEPODIDS MAINTAINED WITH ASSE

2.28.1 Measurement of Copepodid Survival

Copepodids were incubated within the ASSE culture model as described in section 2.27. Wells were observed at 24 hour intervals at x20 magnification and the number of surviving individuals noted according to the criteria of section 2.16. Survival data were collected from 4 normal culture cycles with ASSE and also from maintenance with CHSE and RTG skin equivalents.

2.28.2 Behaviour of Cultured Copepodids

Copepodids were incubated with ASSE, chinook salmon skin equivalent (CHSE) and rainbow trout skin equivalent (RTG) *in vitro* culture models (section 2.25), and skin freshly excised from adult Atlantic salmon (section 2.28.2a). Duplicate 12 well tissue culture plates were prepared for each of the culture substrates as described in section 2.25.

Following disinfection (section 2.27.1) 10 copepodids were added to all wells in 2ml of sterile seawater (33±2‰). Copepodids were also added to 2 additional plates which contained no culture substrate (plastic). Plates were maintained within humid boxes at 10°C. Observations

of copepodid behaviour began following 12 hours of acclimatisation to the culture environment and thereafter at 20h, 30h, 44h, and 60h post incubation.

Observations were made of 2 individuals from duplicate wells of each substrate at each time point. Behaviour was categorised as either swimming, burrowing or settled and measurements made of the duration of each activity (in seconds) of each individual within a 10 minute observation period. Figure 1.3 shows the components of louse anatomy and morphology referred to in the descriptions below.

Swimming: maintenance in water column by burst swimming upwards followed by passive sinking. Contact with the culture substrate was brief and infrequent.

Burrowing: copepodid had contact with culture surface with leading edge of cephalothorax angled down and with posterior segments elevated. Rapid movement of maxillipeds and swimming legs was observed apparently pushing louse forward into substrate, and lunging/grappling movements of second antennae both anteriorly and laterally were also seen . Short periods of inactivity (4-5 seconds) between active burrowing with louse occupying posture of cephalothorax angled down towards the substrate and the first swimming legs positioned laterally extending beyond the margins of the cephalothorax were still regarded as part of the burrowing behaviour. Extended periods of inactivity were classified as settled behaviour.

Settled: copepodid had contact with culture substrate with infrequent movement of appendages. The posterior region was normally parallel to the surface but was occasionally observed with the elongate segments of the posterior region slightly curled beneath the thoracic segments. The leading edge of the cephalothorax may be embedded in the culture substrate with the second antennae often positioned anteriorly, hooked into or through the substrate surface.

2.28.2a Preparation of Excised Salmon Skin

Excised salmon skin was removed from euthanised adult fish (>750g) following surface disinfection with 70% ethanol. Using a dermatome scalpel blade (Swann Morton, UK) parallel incisions of 8-10cm and penetrating approximately 3-4mm were made in the dorsal anterior skin. The incisions were irrigated with HBSS containing 5% v/v Penicillin/Streptomycin solution (Sigma) and 1% v/v 50mg/ml gentamicin and the tissue between the incisions was removed by scalpel strokes made parallel to the surface of the fish. The tissue was mixed for 60

min in the irrigation media used above. The tissue was then removed and rinsed in a stream of sterile 0.01M PBS pH7.4, and subcutaneous tissue dissected away. Tissue of the required diameter were cut and placed in the bottom of culture wells and collagen solution (section 2.20) introduced between the edge of the tissue and the culture well to create a 'seal'. Following polymerisation of the collagen the tissue was maintained in sterile seawater (33±2‰).

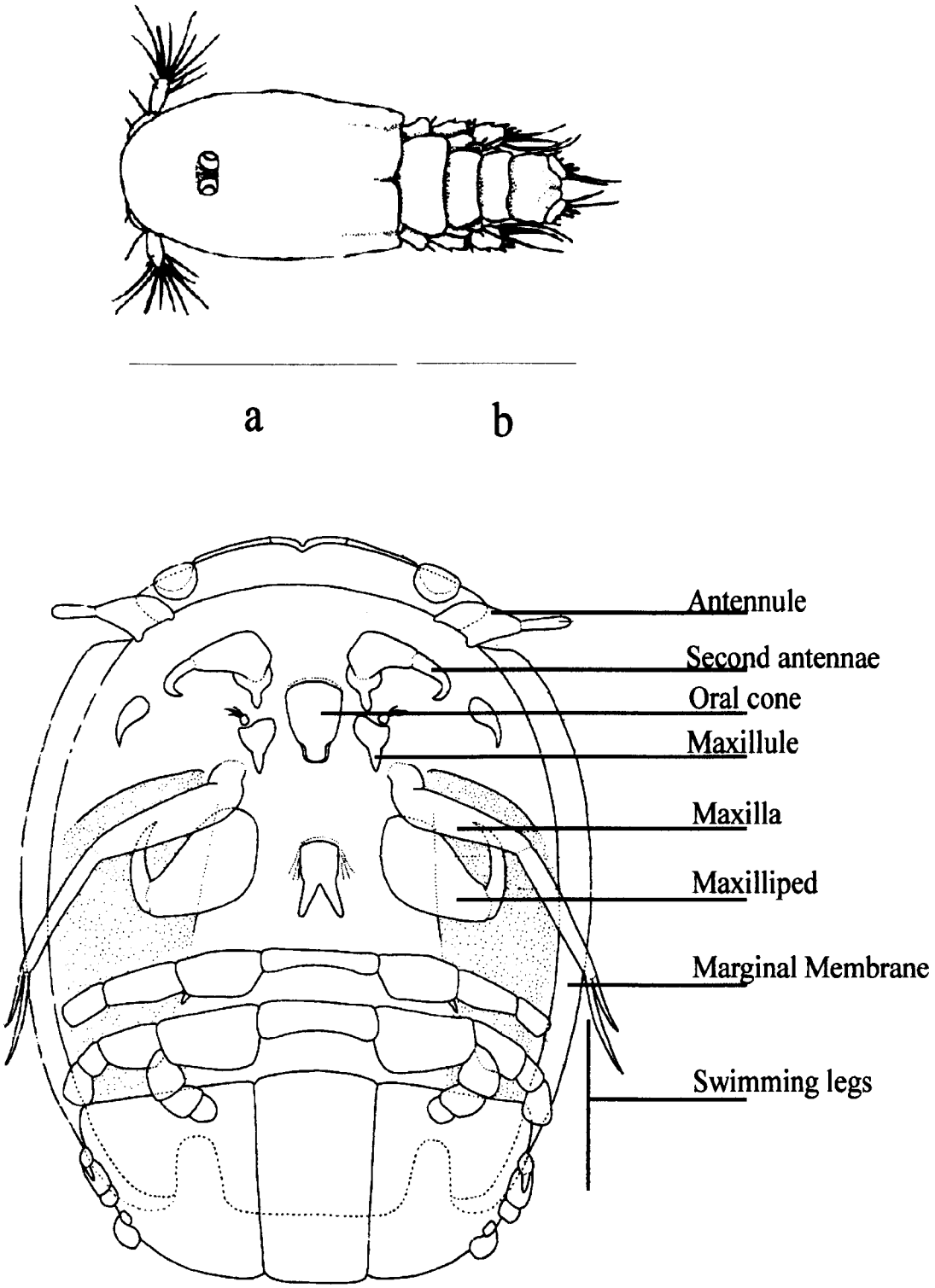

Figure 1.3. Diagram of copepodid anatomy to enable correct identification of appendages for classification of louse behaviour in culture. **Top:** generalised view of copepodid morphology identifying: **(a):** the cephalothorax, composed by the fusion of the cephalic and first two thoracic segments, and **(b):** the posterior region comprising the 3,4,5th thoracic segments (anteriorly) and the elongate segment formed by the fusion of the genital segment and abdomen (posteriorly) (Johnson & Albright, 1991). **Bottom:** Ventral view of adult *L. salmonis* showing arrangement of thoracic appendages used to establish the criteria of behavioural categories (after Kabata, 1979).

2.28.3 Measurement of Feeding Behaviour of Cultured Copepodids

Carmine red was incorporated into the surface collagen layer of ASSE as described in 2.28.3a. Duplicate 24 well culture plates were prepared as for normal culture cycles and 5 disinfected copepodids added to the relevant wells as described in 2.27. All wells were observed daily. ASSE only wells (MC) were monitored for 'leaching' of dye from the surface and for any effects of carmine red on the substrate integrity. Carmine red suspension was added to the seawater media of the LC wells (copepodids in seawater) to monitor for occurrence of dye in guts of non-substrate associated lice. LCS wells were observed to determine incidence of feeding by lice. Presence of the red dye was easily visualised in the gut of feeding animals using a dissecting microscope x40 magnification. The number of lice with dye in gut was noted, as was the number of surviving lice.

2.28.3a Incorporation of Carmine Red into ASSE

A suspension of 4mg carmine red dye (Sigma) was made in 10ml 0.01M PBS pH7.4. The suspension was sterilised by autoclave at 121°C for 20 min. Dye particles were repeatedly washed by centrifugation at 600g for 10 minutes until the remaining particles were no longer solubilized on the addition of 0.01M PBS pH7.4. The suspension was added to the tropocollagen solution at 10% v/v and then polymerised by the addition of sodium bicarbonate and HBSS as described in section 2.20.

2.28.4 Measurement of Length of Cultured Copepodids

Thirty copepodids maintained with ASSE and 30 copepodids from control wells with no cellular substrate were removed from culture and fixed in 8% buffered formal saline. Individuals were first removed on day 3 of incubation and thereafter at 24 hour intervals. Fixed specimens were viewed using Olympus BX60 compound microscope at x100 magnification and measured using calibrated eye piece graticule and the length recorded in millimetres.

2.29 Effects of Supplementation of ASSE on Copepodid Performance

Salmon mucus (section 2.29.1), peptone (section 2.29.2) and DL-methionine (Sigma) were each incorporated into the surface collagen layer of ASSE. All culture controls were included on each plate. Duplicate plates were maintained in duplicate culture cycles using copepodids from 2 separate collections. Mucus aliquots were pooled and incorporated into collagen at 10% and 20% v/v. Peptone was added at 1%, 10% and 15% v/v, and DL-methionine was introduced at 100μg/ml of collagen. Each was added to the tropocollagen stock suspension prior to polymerisation and the gels formed as described in section 2.20. Feeding experiments were also conducted as part of these culture cycles and carmine red was incorporated into surface collagen as described in 2.28.3. Observations of copepodid survival, behaviour and feeding activity were made at 24 hour intervals for the duration of each culture cycle. Behavioural measurements were not performed although qualitative assessment of types of behaviour and the general patterns of louse behaviour were made.

2.29.1 Collection of Salmon Mucus

Six adult Atlantic salmon, euthanised by percussive stunning for extraction of head kidney macrophages, were placed in individual plastic bags prior to dissection. The surface of the fish was rubbed for several minutes to encourage secretion of mucus. The fish were removed and 30ml of 0.01M PBS, pH7.4 added to each bag. Following a period of mixing the PBS/mucus suspension was decanted into centrifuge tubes and mixed for 1 hour at 10°C. Tubes were then centrifuged at 600g for 10min at 4°C and the supernatant collected, filtered (0.22μm) and stored in liquid nitrogen in 1ml aliquots. Protein estimation was performed on 1 aliquot from each fish according to the method described in section 2.15.

2.29.2 Production of Salmon Peptone

This method is based on that first reported by Lewis (1916). A 1Kg adult Atlantic salmon was euthanised by percussive stunning and the internal organs removed. The carcass was chopped into 30-50g pieces and added to 2l of distilled water. The mixture was boiled for 60 min with continual stirring and then drained through a domestic sieve. The liquor was retained and the tissue homogenised and boiled for an additional 30 min with 1l of distilled water. After

sieving, the liquor was pooled and allowed to cool and the debris to sediment. The supernatant was filter sterilised to 0.22μm and stored at 4°C.

2.30 The Use of Fish Skin Equivalents in The Study of Host Selection by *L. salmonis* Copepodids

Thirty ml of 1% agarose (Sigma) was added to four replicate sterile petri dishes (Sterilin). After cooling, 4 wells with a diameter of 2.5cm were cut into the agarose and the agarose discs discarded. An additional well of 2.5cm diameter was cut but the agarose kept in place. Culture substrates (ASSE, CHSE and RTG) were prepared as described in section 2.25 using 12 well tissue culture plates. When fully formed the substrates were carefully lifted from their culture wells and placed (maintaining their orientation) into a well of the petri dish. One well was left with no substrate (plastic). Collagen was added between the circumference of each substrate and the surrounding agarose to form a 'seal'. Following polymerisation of the collagen the surface was washed in a stream of sterile 0.01M PBS pH7.4 and 20ml of sterile seawater added. One hundred and twenty copepodids, previously maintained according to section 2.1.3, were added to each replicate petri dish and incubated at 10°C in 12d:12l photoperiod, and distributed randomly with respect to the light source. The experiment was monitored at 6h, 12h and 24h post incubation and thereafter at 24 hour intervals. The number of copepodids observed in contact with each culture substrate was noted; those in contact with the agarose boundaries were not counted. Controls included duplicate petri dishes with 5 wells each containing the same substrate to determine the suitability of each within this configuration, to copepodid settlement. In a separate experiment the substrates were also modified by the addition of 0.1mg/ml salmon mucus and 1% v/v peptone (section 2.29), except the wells containing agarose or no substrate. This experiment was conducted to the same method described above.

The Effects of Culture Supernatants On Salmon Macrophage Immune Functioning

Macrophages, isolated from head kidney of 16 adult Atlantic salmon following the method described in section 2.8 were incubated with culture supernatants for 24 hours (section 2.31). The reproducibility of results was tested by using supernatants from 3 separate culture cycles using samples collected on days 1,2,3,6 and 10 of each cycle. Supernatant treated macrophages

were employed in assays to measure intracellular respiratory burst (section 2.10), phagocytosis (section 2.9) and chemotaxis (section 2.11).

2.31 INCUBATION OF MACROPHAGES WITH CULTURE SUPERNATANTS

Culture supernatants were removed from liquid nitrogen storage and their protein concentration calculated as described in section 2.5. The protein concentration was adjusted to 0.4mg/ml with 0.01M PBS pH7.4. Seawater samples were used 'neat' since they possessed no measurable protein content. The macrophage suspension was adjusted to the cell density required by the chosen cellular assay using L-15 (washing) and 1ml aliquots of this suspension were incubated with 500µl of the culture supernatants for 24 hours at 10°C with continuous mixing. The suspension was then centrifuged at 400g for 10 min and the cell pellet resuspended in 1ml of L-15(washing). These 'treated' macrophages are then ready for use in cellular assays.

2.32 NORMALITY OF MACROPHAGES TREATED WITH CULTURE SUPERNATANTS

In order to determine the effects of incubation with culture supernatants on macrophages, and the potential for effecting the outcome of cellular assays, the following parameters of treated and untreated cells were investigated. For the purposes of these measurements cells from duplicate Atlantic salmon were incubated for 24 hours with each culture supernatant at various dilutions following the method given above. Supernatants were selected from culture cycle 3 and were pooled from days 3,4 and 5 of the culture. Controls consisted of untreated macrophages.

2.32.1 Macrophage Morphology

Monolayers of macrophages were prepared on glass slides and viewed x100 oil immersion. Duplicate slides were made for each fish and the longest axis of 10 cells per slide was measured using an eye piece graticule.

2.32.2 Macrophage Adherence

One hundred microlitres of a $1x10^7$/ml macrophage suspension was added to duplicate wells of 16 well chamber slides (Nunc). Cells were allowed to adhere overnight and then washed 3 times in 0.01M PBS pH7.4. Cell number was determined from duplicate counts of cells in each well using the method described in section 2.14.

THE PRELIMINARY BIOCHEMICAL PROPERTIES OF CULTURE SUPERNATANTS

2.33 THE PROTEIN CONCENTRATION OF CULTURE SUPERNATANTS

Following harvest from culture wells and prior to storage in liquid nitrogen the protein content of supernatants was calculated using the method in section 2.5. The assay was performed on samples collected from every day of incubation from duplicate wells of each culture group (LC, MC, LCS, SW) on each plate from 2 of the 4 normal culture cycles (since the requirement for stored supernatants in additional assays did not permit the use of samples from all cycles) .

2.34 EFFECTS OF HEATING AND DILUTION ON THE IMMUNOLOGICAL EFFECTS OF CULTURE SUPERNATANTS

Culture supernatants (LC, MC, LCS and SW) from days 1,2,3,6 and 10 of culture cycle 3 were removed from liquid nitrogen storage and assayed for protein content (section 2.5). The protein concentration of each was adjusted to 0.4mg/ml with 0.01M PBS pH7.4 (those samples with protein levels below this were used neat). Each supernatant was divided into 3 aliquots which were either heated to 40°C for 20 minutes, heated to 80°C for 20 minutes, or remained unheated. Supernatants were then incubated with macrophages from 4 adult Atlantic salmon (section 2.31) and incorporated into the chemotaxis assay (section 2.11).

Culture supernatants corresponding to days 1,2,3,6 and 10 of culture cycle 4 were thawed and protein content adjusted as described above. Supernatants were likewise divided into 3 aliquots

and diluted in PBS to 100x and 1000x with one aliquot remaining undiluted. The supernatants were then incubated with macrophages and used to measure chemotaxic ability.

2.35 Effects of Proteinase K Digestion on The Immunological Effects of Culture Supernatants

Dialysed culture supernatants were removed from liquid nitrogen storage and thawed at room temperature. Fifty microlitres of 10mg/ml Proteinase k (type XI) (Sigma) was added to 1ml of culture supernatant and mixed for 1 hour at room temperature. Proteinase k was inactivated by the addition of 20µl 0.1M Phenylmethanesulfonyl fluoride (PMSF, Sigma) (2mM final concentration). The solution was mixed for 2 hours at room temperature which permitted sufficient time for PMSF inactivation of Proteinase k and for PMSF to become inactive. Following protein digestion, the culture supernatants were incubated with head kidney macrophages from 4 adult Atlantic salmon as described in section 2.31 and these cells used in chemotaxis assays (section 2.11)

2.36 Determination of Enzyme Profiles of Supernatants (APIZYM)

Semi-quantitative analysis of enzymatic activity of culture supernatants was performed using APIZYM test strips (Biomereux). Tests were conducted according to manufacturers instructions for supernatants from culture cycles 2 and 3 collected on day 1,2,8 and 10. Briefly, 65µl of undiluted supernatants (following dialysis, filtration and storage in liquid nitrogen) were added to each of the 20 substrate containing wells of the test strip. Strips were incubated for 4 hours at 37°C following which the reactions were resolved by the addition of a single drop of ZymA and ZymB reagents. The magnitude of each reaction was determined by colourmetric comparison with a standard chart which allows assignment of scores from zero, no reaction, to 5, maximum intensity.

The same test was also applied to a homogenate of 40 free-swimming copepodids. These were removed from stock culture on day 2 and day 6 post metamorphosis to copepodid and homogenised by vortexing with sterile glass beads. Particulates were removed by centrifugation and the supernatant retained and added to the APIZYM test strips as described above.

2.37 The Use of Gel Filtration Chromatography to Determine Molecular Size of Proteins Within Culture Supernatants

Unlike electrophoretic techniques, gel filtration allows the separation, and weight and size determination of native or denatured globular proteins irrespective of pH, ionic strength or temperature. The sample is loaded at the top of columns packed with gels of cross-linked polymers such as dextran with a known and controllable pore size. An elution buffer with pH and ionic strength suitable to the preservation of the sample is pumped from the top of the column. Large molecules are eluted early in the chromatographic separation whilst smaller molecules diffuse into the porous gel and their passage through the column is slowed. Thus, the sample components of higher molecular weight are eluted first followed by successively smaller molecules. The speed of the elution process directly affects the effectiveness of the separation with slower speeds giving better resolution. Using the ÄKTA™ FPLC system (Amersham Pharmacia Biotech) the eluent can be simultaneously analysed for conductivity, pH and UV absorption and the results plotted on chromatograms, and the separated fractions collected.

ASSE culture supernatants (LC, MC, LCS and SW) from day 5 of culture cycles 1 and 4 were removed from liquid nitrogen storage. The protein concentration was calculated (section 2.5) and adjusted to 0.4 mg/ml in an elution buffer containing 50mM potassium phosphate and150mM sodium chloride pH7.97. The louse control supernatant (LC) was first concentrated using VectaSpin® bioconcentrators (Whatmann) with 5000 Daltons molecular weight cut off. Seawater supernatants were used neat since they had no detectable protein concentration. One hundred microlitres of each sample was loaded onto a Supadex S200 HR 10/30 gel filtration column (Amersahm Pharmacia Biotech) connected to an ÄKTA™ FPLC. The sample was eluted from the column in elution buffer at 400µl/minute. The eluent was passed through a UV monitor and 5ml fractions collected (Frac-901, Amersham Pharmacia Biotech). The change in UV absorption of the eluent was plotted as a chromatogram (absorption (mAU) against eluent / sample volume (ml)). One column volume of elution buffer was used between application of samples to equilibrate the column.

Chromatographic peaks were distinguished using Unicorn™ v3.0 software (Amersham Pharmacia Biotech). A peak was defined as a minimum change in the height of the absorbance curve of 0.01mAU per 0.001ml sample volume. Small changes in absorbance that meet these criteria may therefore be registered as peaks that might not be easily discernible as such on the chromatograms.

The column was calibrated by loading a 100µl sample containing 0.7mg.ml ferritin (440 Kilodaltons, KDa), 2mg/ml aldolase (150 KDa), 2mg/ml albumin (66 KDa), 2mg/ml ovalbumin (43 KDa) and 2mg/ml ribonuclease A (13.7 KDa). The calibration curve was applied to the sample absorption data using Unicorn™ v3.0 software which allowed determination of the molecular weights of the protein peaks shown on the chromatograms. The software also allowed subtractive analysis of multiple chromatograms to determine the presence of corresponding peaks as well as identifying peaks not found in the other samples.

2.38 The Effects of Chromatography Fractions on the Immunological Functioning of Salmon Macrophages

Those fractions of LCS supernatants collected following gel filtration that were not seen in chromatograms of LC or MC supernatants were assayed for their effects on macrophage chemotaxis. Macrophages were isolated from the head kidney of 2 adult Atlantic salmon (section 2.8) and incubated with 6 individual fractions following the method in section 2.31. These fractions were selected because they displayed the most obvious or largest peaks on chromatograms. Equal volumes of all 14 fractions identified as 'unique' to LCS supernatants were also pooled and incubated with macrophages. Fractions from both MC and LC supernatants were similarly pooled and incubated with macrophages. Following treatment the macrophages were assayed for their chemotaxic responses using the method described in section 2.11. The protein content of each fraction was also calculated prior to incubation with macrophages using the method in section 2.5.

2.39 Statistical Analysis

All statistical analyses were carried out using SYSTAT. Data are presented as means and standard deviation or as percentages. Where appropriate data were compared by paired two-sampled t-test and by one way analysis of variance (ANOVA) to identify sample groups which were significantly different at the 5% level. Statistical comparison of chromatograms from FPLC analyses were performed using Unicorn™ v3.0 software (Amersahm Pharmacia Biotech).

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THE COMPARATIVE IMMUNOLOGICAL EFFECTS OF *L. SALMONIS* INFECTION OF ATLANTIC SALMON AND RAINBOW TROUT

Chapter 3

INTRODUCTION

THE COMPARATIVE IMMUNOLOGICAL EFFECTS OF *L. SALMONIS* INFECTION OF ATLANTIC SALMON AND RAINBOW TROUT.

The casual observation that coho salmon co-cultured at the same site with Atlantic salmon in Canada suffered lowered burdens of *L. salmonis,* was first tested scientifically by Johnson and Albright (1992). In this experiment they were able to reproduce these field observations by demonstrating that Atlantic salmon did indeed become more heavily infested with *L. salmonis* than either coho or chinook salmon when exposed to the same dose of infective copepodids. They found that 15 days post infection coho salmon had significantly fewer lice than Atlantic salmon or chinook salmon and that the copepods infecting coho salmon had significantly reduced developmental rates. Furthermore, histological examination of the parasite attachment sites showed extensive epithelial hyperplasia, encapsulation of the infecting larvae and a well developed inflammatory response characterised by influx of neutrophils, macrophages and lymphocytes in the first days of the infection. By comparison, the tissue responses of Atlantic salmon were described as mild and inadequate to prevent the parasite from causing extensive epithelial erosion. These findings agree with those reported by Jones *et al.* (1990) who described the lack of dermal reaction to larvae feeding on Atlantic salmon.

The extent of the apparent suppression of cellular immune responses, whether it is localised or systemic, was not further investigated by these authors. However, more recent work by Mustafa *et al.* (2000) has pointed to a likely system-wide immunosuppression caused by *L. salmonis* infections. The authors collected macrophages from the head kidney of Atlantic salmon during a sea louse infection and measured their phagocytic and oxidative defence responses in standard immunological assays. Whilst a reduction in the percentage of cells undergoing respiratory burst began to fall from day 7 of the infection it had not significantly fallen until day 21 by which time the phagocytic ability of the cells from infected fish was also significantly reduced. However, these results correlate to increases in blood glucose and cortisol levels also recorded during the infection. The question remains whether the reduced systemic immunity was due to active louse immunosuppression hypothesised by Johnson and Albright in their observed localised effects, or simply caused by physiological changes induced by the stress responses of the chronically infected fish. Systemic immunodepression by arthropod parasites is reported in

many cases of mammalian parasitism (Wikel *et al.*, 1994; Wikel *et al.*, 1996) but is indicated only by largely inconclusive evidence in fish presented by the above authors.

The question of the comparative susceptibility of hosts to the same parasite involves aspects of the evolutionary biology of both parasite and host and the subject of host specificity. For the most part it is believed that even when a parasite can develop in multiple primary hosts it usually performs best in a single host and less readily in the rest (Cox, 1983; Cox, 1984). Typically parasite and host share evolutionary history so that ancestral parasites of an animal will develop to become the parasite of the modern descendants. The true parasitic relationship that develops is one whereby the parasite adapts to allow its maintenance within or upon the host without causing pathological changes in the host. Parasitism of a non niche species is either not acceptable to the parasite, or results in negative effects upon the host. *C. elongatus* is known to parasitise approximately 37 species of fish in UK waters alone (Kabata, 1979), *L. salmonis* on the other-hand is reported only from salmonid species. Evidence from fish farming and from natural infections of wild Pacific salmon however suggests that *L. salmonis* appears to have preferred host species within its range of potential hosts. Whilst capable of completing their life cycle on all of the 6 species of Pacific salmon examined by Nagasawa *et al.* (1993) greater than 90% were distributed on pink and chum salmon. Similarly, Jackson *et al.* (1997) showed that both the intensity and abundance of *L. salmonis* on rainbow trout in Ireland was consistently lower than on Atlantic salmon when the species were co-cultured. The comparative susceptibility of Atlantic salmon and sea trout (*Salmo trutta*) was investigated in tank trials by Dawson *et al.* (1997) who concluded that in mixed species populations *L. salmonis* selectively settled on sea trout. They argued that Atlantic salmon may still represent the parasite's niche host since the intensity of the parasitism on this species was low, as was the epidermal damage to the fish, suggesting a balance between the parasite and the hosts immune response. The counter argument would be that in extreme conditions, such as experienced during tank trials, any interactive balance between parasite and host is lost so that the apparent 'preference' for the sea trout host may actually indicate its true niche species. They speculated on the improved immunological responses of Atlantic salmon towards the parasite but did not go on to examine this. In fact the comparative immunological responses of susceptible species have not been investigated with respect to this parasite which is the objective of this chapter.

MATERIALS AND METHODS

This chapter details an experiment in which Atlantic salmon and rainbow trout maintained in mono- and co-culture were simultaneously infected with *L. salmonis* copepodids (section 2.2) and sampled at time intervals following infection. At each time point the developmental stage and population size *L. salmonis* was recorded and samples of peripheral blood (section 2.3) and head kidney (section 2.8) removed.

The physiological status of the fish was determined by calculating the packed cell volume (section 2.3.1) and total serum protein concentration (section 2.5) of the sampled blood, whilst indication of the non-specific humoral immune response to the infection was determined by measurement of the serum lysozyme activity (section 2.6). Blood samples were also used to investigate the specific humoral immune responses to the infection by measuring agglutinating antibody production towards *L. salmonis* antigens (section 2.7.1) and *Aeromonas salmonicida* (section 2.7.2). The cellular immunity of infected fish was measured by calculation of circulating leucocyte populations (section 2.3.2), and phagocytosis (section 2.9), respiratory burst (section 2.10) and chemotaxis (section 2.11) of macrophages isolated from infected and non-infected fish.

The data were statistically compared both between species and between culture condition (either mono or co-culture) using paired two-sampled t-test and one way analysis of variance where appropriate.

RESULTS

Comparison of the Susceptibility of Atlantic salmon and rainbow trout to Infection by *L. salmonis.*

At each sample point of the challenge, 10 fish per species per tank were examined and the number and developmental stage of *L. salmonis* recorded. Data in figures 3.1 and 3.2 are presented as means and standard deviation, and as percentages. One-way analysis of variance was used to compare the mean number of lice within and between mono-cultured and co-cultured groups of each species.

Figure 3.1 shows the mean total number of lice per fish. Infected salmon and trout maintained in mono-culture were equally susceptible to louse infection. There was no significant difference in the total louse burden of each group with a mean number of approximately 75 lice per fish over the first 12 days of the challenge. This burden declined to 30 lice per fish during the second half of the experiment. The rate of development and occurrence of each developmental stage was not significantly different between these two groups. At day 3 post challenge the population was predominately composed of copepodids, day 6 by chalimus I, chalimus II and III on day 12, chalimus IV on day 18, chalimus IV and pre-adult I on day 25 and by pre-adult I and II on the final day of sampling. This pattern in the proportional composition of the total louse population was also repeated in the co-cultured challenged groups. During the course of the infection, the louse population dynamics were not influenced significantly by either species of culture condition.

However, the total louse population did vary significantly between co-cultured challenged groups and between trout groups. From day 6 until the end of the challenge, except on day 25, the number of lice infecting co-cultured salmon was significantly higher than on mono-cultured salmon (approximately 23% higher on day 6). Salmon also suffered significantly higher sea lice burden when co-cultured with trout. In this case trout appear less susceptible to infection than salmon since the difference in total louse numbers was significant for the duration of the

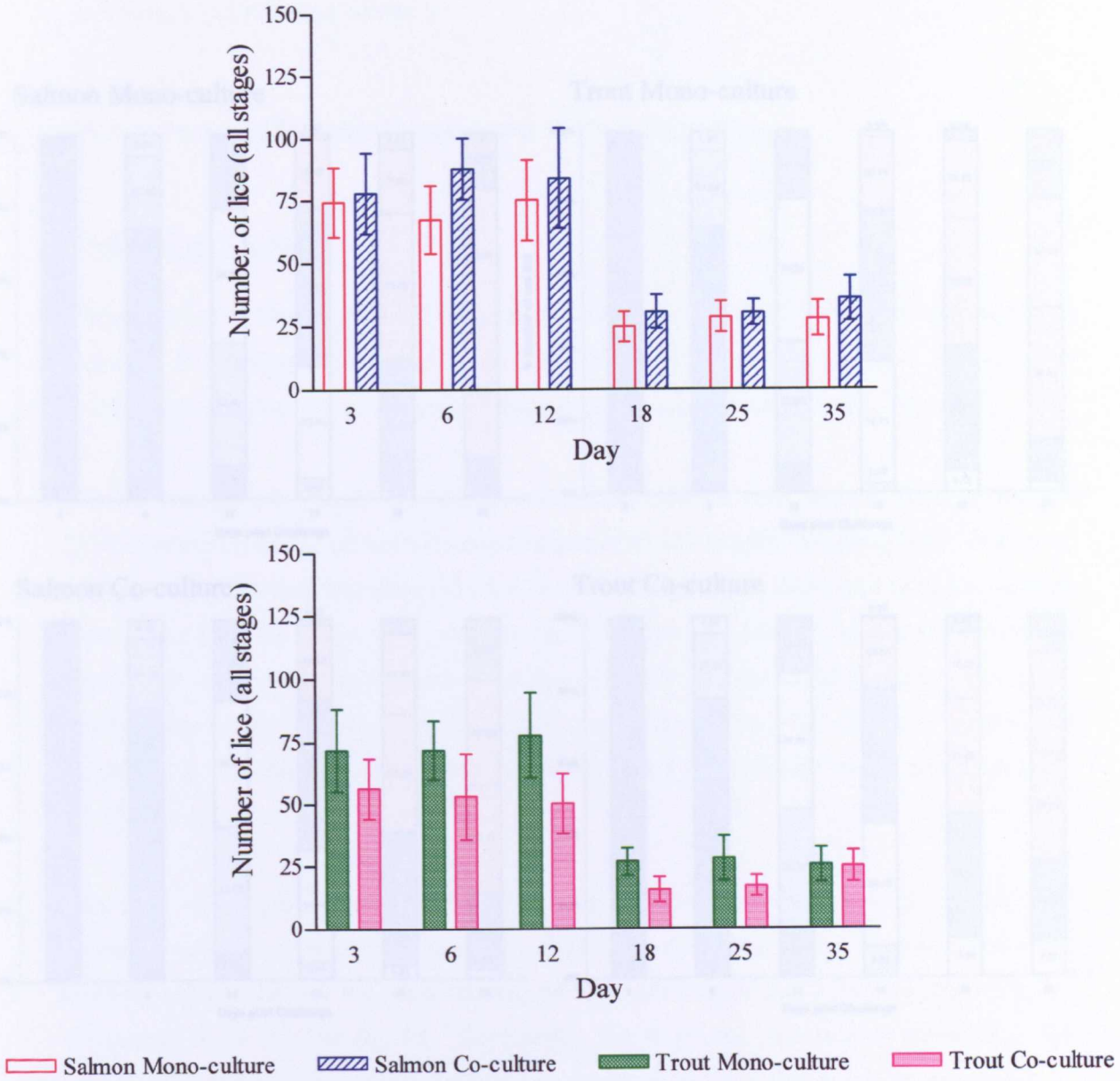


Figure 3.1

Mean number of sea lice (all stages) (± standard deviation) infecting Atlantic salmon and rainbow trout following experimental infection. At each sample point n=20 fish per group.

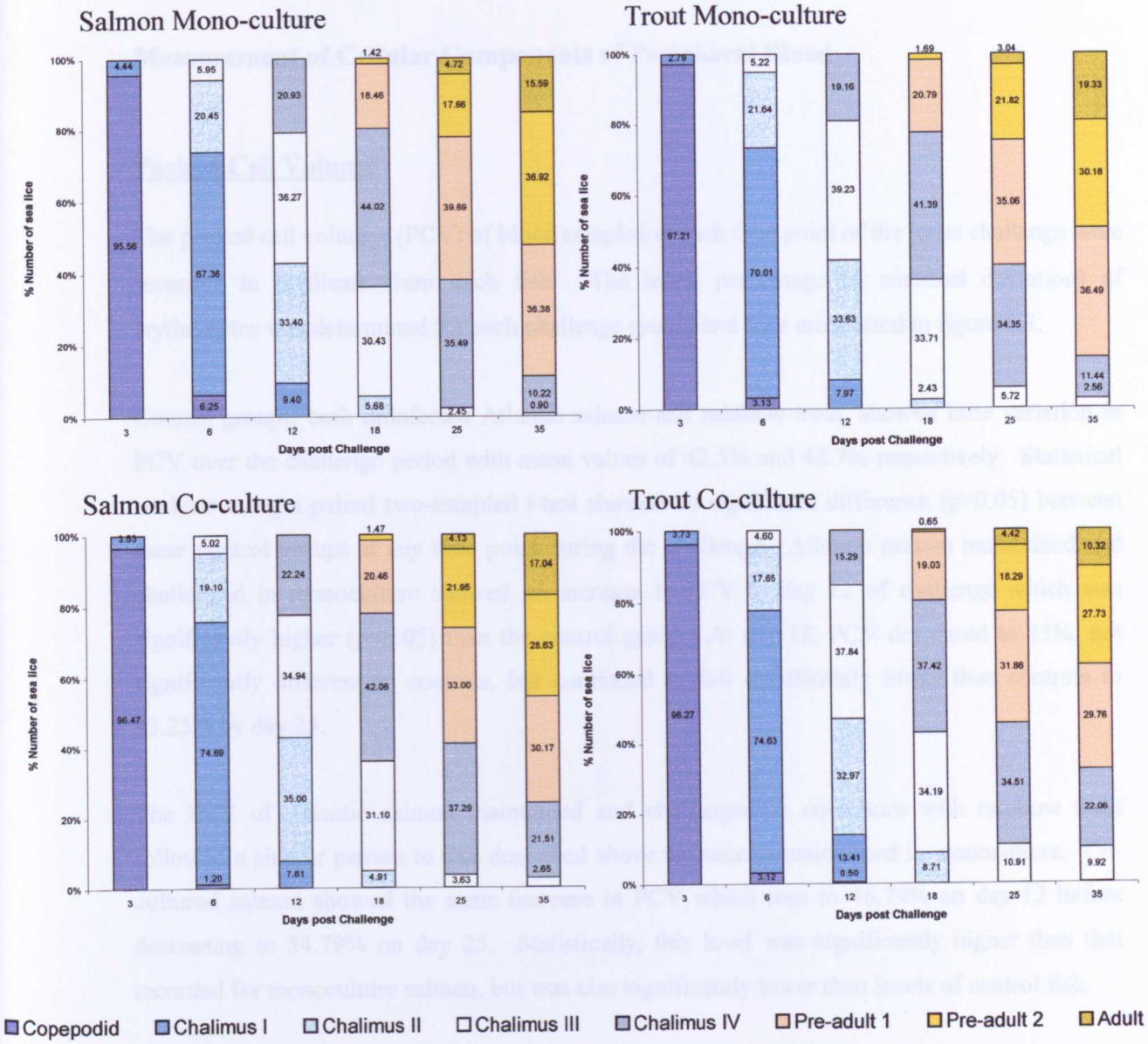


Figure 3.2

Percentage developmental stage composition of sea lice population on Atlantic salmon and rainbow trout maintained in mono and co-culture following experimental infection. At each sample point n=20 fish per group. The total number of lice observed can be determined by reference to Figure 3.1.

experiment. The greatest difference occurred on day 6 when the population infecting salmon was 40% greater than that on rainbow trout maintained in the same tank. From day 3 until day 25 of the challenge, co-cultured trout were infected with significantly fewer numbers of sea lice, approximately 35% lower on day 12.

Measurement of Cellular Components of Peripheral Blood

Packed Cell Volume

The packed cell volumes (PCV) of blood sampled at each time point of the louse challenge were recorded in duplicate from each fish. The mean percentage (± standard deviation) of erythrocytes was determined for each challenge group, and they are plotted in figure 3.3.

Control groups, both uninfected Atlantic salmon and rainbow trout, showed little variation in PCV over the challenge period with mean values of 42.5% and 42.7% respectively. Statistical analysis using a paired two-sampled t-test showed no significant difference ($p>0.05$) between these control groups at any time point during the challenge. Atlantic salmon maintained and challenged in monoculture showed an increase in PCV to day 12 of challenge which was significantly higher ($p<0.05$) than the control group. At day 18, PCV decreased to 43%, not significantly different to controls, but continued to fall significantly lower than controls to 33.25% by day 25.

The PCV of Atlantic salmon maintained and challenged in co-culture with rainbow trout followed a similar pattern to that described above for salmon maintained in monoculture. Co-cultured salmon showed the same increase in PCV which rose to 46.79% on day 12 before decreasing to 34.79% on day 25. Statistically, this level was significantly higher than that recorded for monoculture salmon, but was also significantly lower than levels of control fish.

Rainbow trout maintained and challenged in monoculture showed no significant deviation from the PCV values seen in the control fish until day 12 when levels in challenged fish rose to 45%. Values continued to increase until day 18 where they were significantly higher than controls at 45.5% before rapidly declining to 33.5% by day 25 (significantly lower than controls). Trout co-cultured and challenged with salmon displayed a markedly different pattern. Following a

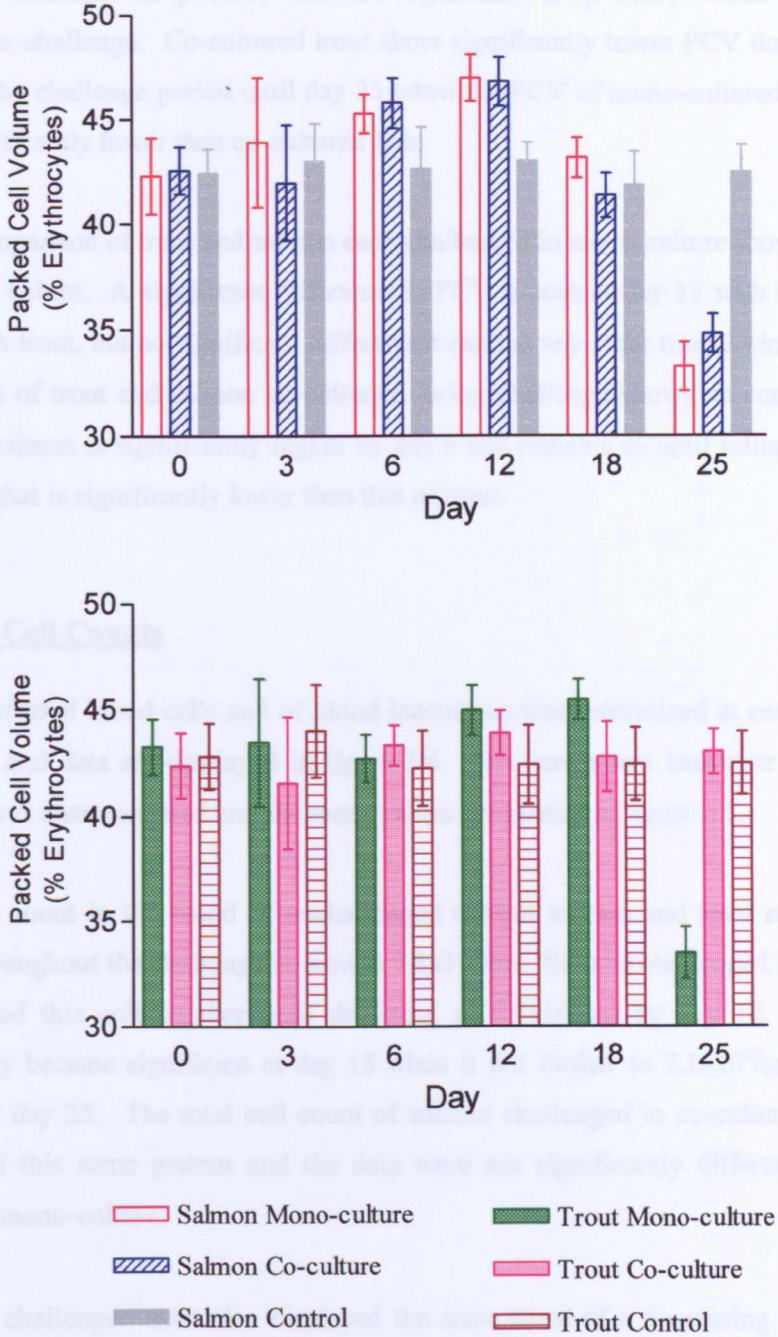


Figure 3.3

Mean packed cell volume (± standard deviation) of blood withdrawn from Atlantic salmon and rainbow trout following experimental infection with *L. salmonis*. Day 0 represents samples collected from the population prior to challenge (n=8, N=24). At all subsequent sample points n=20, N=80.

n = number of fish sampled per group
N = total number of measurements made per group

significant decrease in PCV at day 3, the values increased to levels slightly above the controls (significantly different at $p<0.05$, but not significant at $p<0.01$) which are maintained throughout the challenge. Co-cultured trout show significantly lower PCV than monocultured trout during the challenge period until day 25 when the PCV of mono-cultured trout decreased to levels significantly lower than co-cultured fish.

Statistical comparison of trout and salmon each challenged in mono-culture shows a comparable trend in PCV values. A significant difference in PCV is seen at day 12 with levels in salmon above those in trout, but no significant differences exist at any other time during the challenge. A comparison of trout and salmon co-cultured during challenge shows no comparable trends. The PCV of salmon is significantly higher by day 6 and remains so until falling rapidly at day 25 to a value that is significantly lower than that of trout.

Differential Cell Counts

The number of total blood cells and of blood leucocytes was determined at each time point of the challenge and data are displayed in figure 3.4. The percentage leucocyte composition of whole blood was also measured and the mean values are plotted in figure 3.5.

The total cell count in the blood of unchallenged control salmon and trout remained largely unchanged throughout the challenge at around $7.8x10^8$/ml. Salmon challenged in mono-culture also maintained this cell number until declining to $7.5x10^8$/ml by day 12. However, the difference only became significant at day 18 when it fell further to $7.1x10^8$/ml and finally to $6.2x10^8$/ml by day 25. The total cell count of salmon challenged in co-culture with rainbow trout followed this same pattern and the data were not significantly different from salmon maintained in mono-culture.

Mono-culture challenged trout also displayed the same trend of a decreasing total blood cell count which also deviated significantly from the control fish at day 18 and fell to $6.6x10^8$/ml by day 25. Co-cultured trout also showed the same decrease in cell number as the challenge progressed and which also became significantly different to control numbers at day 18. However, numbers of cells declined only to $6.9x10^8$/ml which was higher than the other challenged groups, although not significantly so.

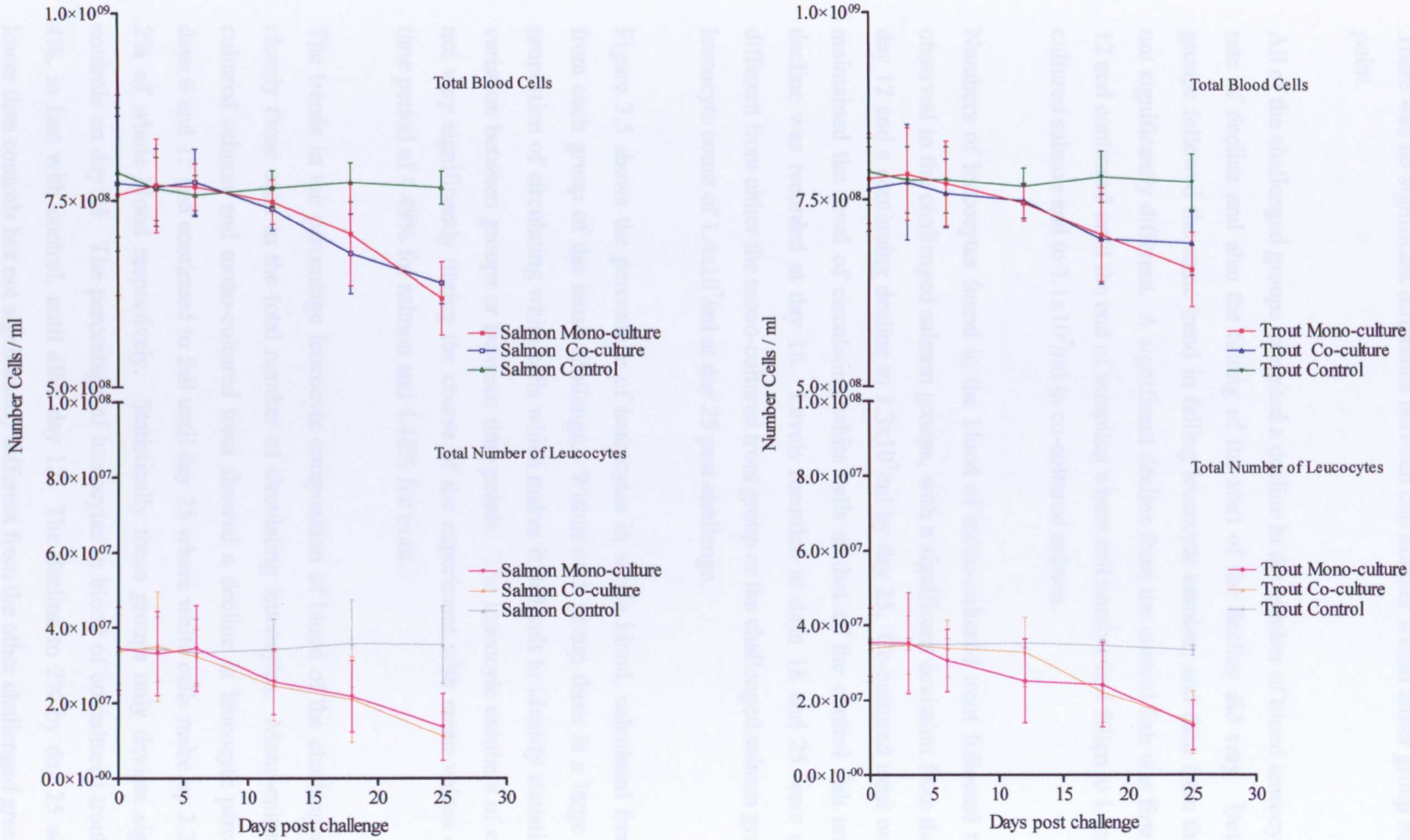


Figure 3.4

Total cell number (mean ± standard deviation) (data above $5x10^8$ cells/ml) and mean number of leucocytes (± standard deviation) (data below $1x10^8$ cells/ml) in blood from Atlantic salmon and rainbow trout following experimental infection with *L. salmonis*. Day 0 represents data from samples collected from the population prior to challenge (n=8). At all subsequent sample point n=20 fish per group.

Numbers of circulating leucocytes remained relatively constant throughout the experiment in both of the uninfected control groups at a mean of approximately $3.5x10^7$/ml, although the salmon control showed a greater variation in number ranging from $2.8x10^7$/ml to $4.4x10^7$/ml. There was no significant difference between cell number within either group between each time point.

All of the challenged groups exhibited a decline in the number of blood leucocytes, although the rate of decline and also the timing of the start of the decline did vary. Both of the salmon groups followed the same trend in falling leucocyte number, and data from these groups were not significantly different. A significant decline from the control fish was first observed at day 12 and continued until the end of sampling where cell number had fallen to $1.4x10^7$/ml in mono-cultured salmon and to $1.1x10^7$/ml in co-cultured salmon.

Numbers of leucocytes found in the blood of mono-cultured trout followed the same pattern observed in the challenged salmon groups, with a significant deviation from the control fish by day 12 and a continuing decline to $1.3x10^7$/ml by day 25. Co-cultured trout on the other hand, maintained the level of circulating white cells at that of the control fish until a significant decline was recorded at day 18. Levels recorded at days 18 and 25 were not significantly different from either the mono-cultured trout group or the challenged salmon groups with a total leucocyte count of $1.4x10^7$/ml at day 25 post challenge.

Figure 3.5 shows the percentage of leucocytes in whole blood, calculated from blood smears from each group of the louse challenge. Within each group there is a large variation in the proportion of circulating white cells which makes it difficult to identify statistically significant variation between groups or between time points. The leucocyte numbers of control fish does not vary significantly during the course of the experiment with mean values over the 25 day time period of 4.49% for salmon and 4.46% for trout.

The trends in the percentage leucocyte composition of blood of the challenged groups follow closely those seen in the total number of circulating leucocytes. Mono-cultured salmon, co-cultured salmon and mono-cultured trout showed a decline in leucocyte percentage between days 6 and 12 and continued to fall until day 25 where white cells make up 2.21%, 1.75% and 2% of whole blood respectively. Statistically these groups only deviate significantly from controls on day 25. The percentage of leucocytes in blood of co-cultured trout remains above 4%, in line with control, until after day 12. The decline to 2% by day 25 was significantly lower than controls but not significantly different from the other challenged groups.

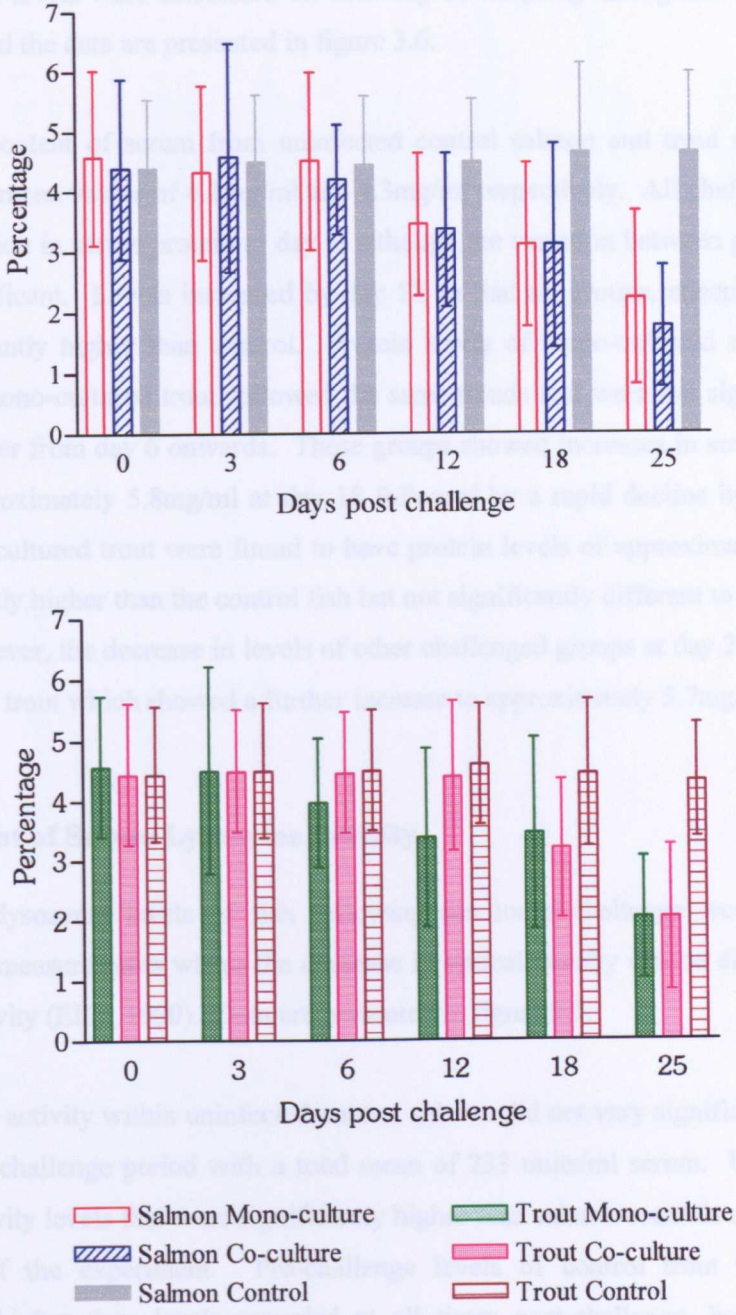


Figure 3.5

Mean percent (± standard deviation) leucocyte composition of blood from Atlantic salmon and rainbow trout following experimental infection with *L. salmonis*. Day 0 represents data collected from the population prior to challenge (n=8). At all subsequent time points n=20 fish per group.

Measurement of Serum Protein Concentration

Serum protein levels were calculated on each day of sampling throughout the louse challenge experiment and the data are presented in figure 3.6.

The protein content of serum from uninfected control salmon and trout remained relatively constant with mean values of 4.1mg/ml and 4.3mg/ml respectively. All challenged fish showed slight depression in serum protein to day 6, although the variation between groups and controls was not significant. Levels increased by day 12 so that all groups, except co-cultured trout, were significantly higher than control. Protein levels of mono-cultured salmon, co-cultured salmon and mono-cultured trout followed the same trends and were not significantly different from each other from day 6 onwards. These groups showed increases in serum protein to peak levels of approximately 5.8mg/ml at day 18 followed by a rapid decline by day 25 to around 3mg/ml. Co-cultured trout were found to have protein levels of approximately 5mg/ml at day 18, significantly higher than the control fish but not significantly different to the other challenge groups. However, the decrease in levels of other challenged groups at day 25 was not observed in co-cultured trout which showed a further increase to approximately 5.7mg/ml.

Measurement of Serum Lysozyme Activity

Total serum lysozyme levels of fish following sea louse challenge were calculated from turbidimetric measurements where the decrease in optical density can be directly correlated to lysozyme activity (Ellis, 1990). Data are presented in figure 3.7.

The lysozyme activity within uninfected control salmon did not vary significantly between time points of the challenge period with a total mean of 233 units/ml serum. Uninfected rainbow trout had activity levels that were significantly higher than salmon controls at 789 units/ml over the course of the experiment. Pre-challenge levels of control trout were found to be significantly higher than levels recorded at all times post-challenge, however, these post-challenge values did not vary significantly between time points as the experiment progressed.

By day 3 of the experiment all challenged groups showed elevated lysozyme activity except infected co-cultured trout which showed a slight depression in activity. These changes did not differ significantly from controls. As the infection continued activity levels in infected salmon groups increased and deviated significantly from controls by day 12 when levels in co-cultured

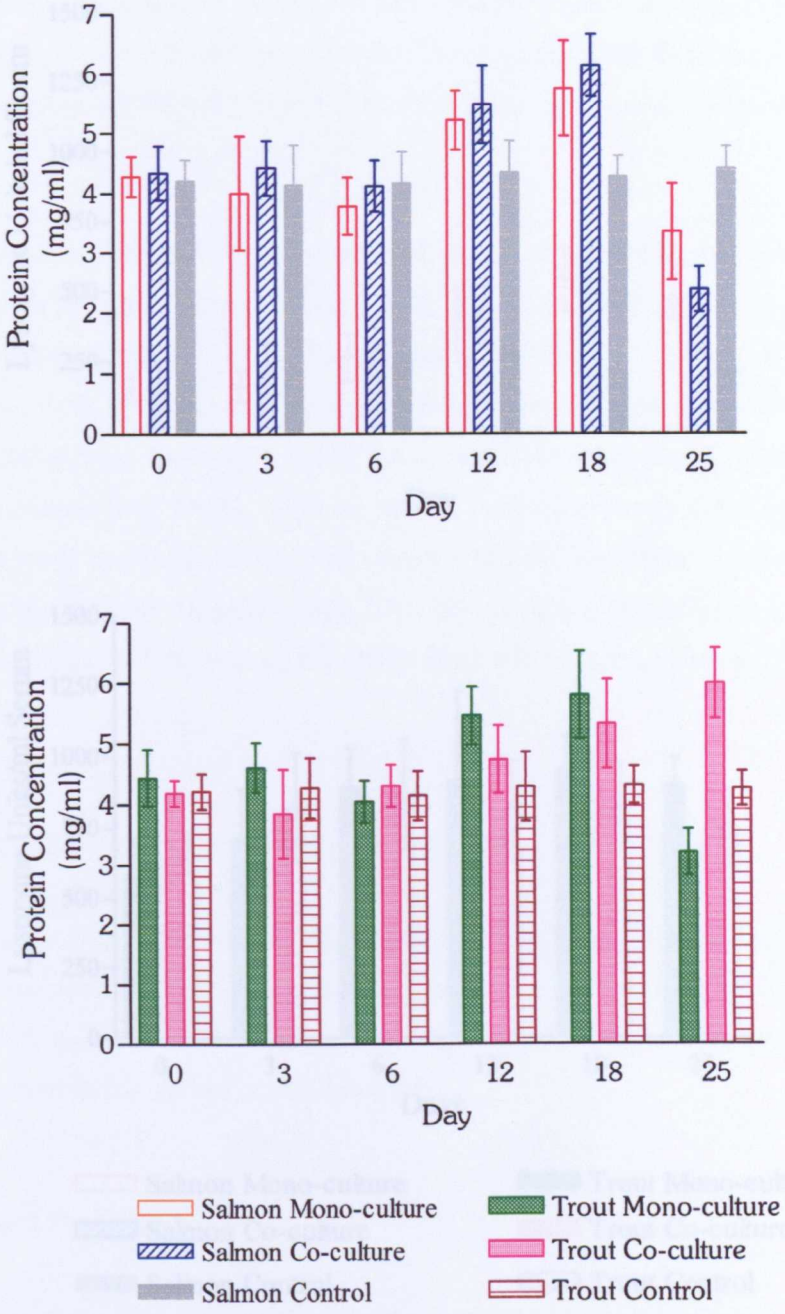


Figure 3.6

Mean (± standard deviation) serum protein concentration (mg/ml) of blood from Atlantic salmon and rainbow trout following experimental infection with *L. salmonis*. Day 0 represents data collected from the population prior to challenge (n=8). At all subsequent time points n=20 fish per group.

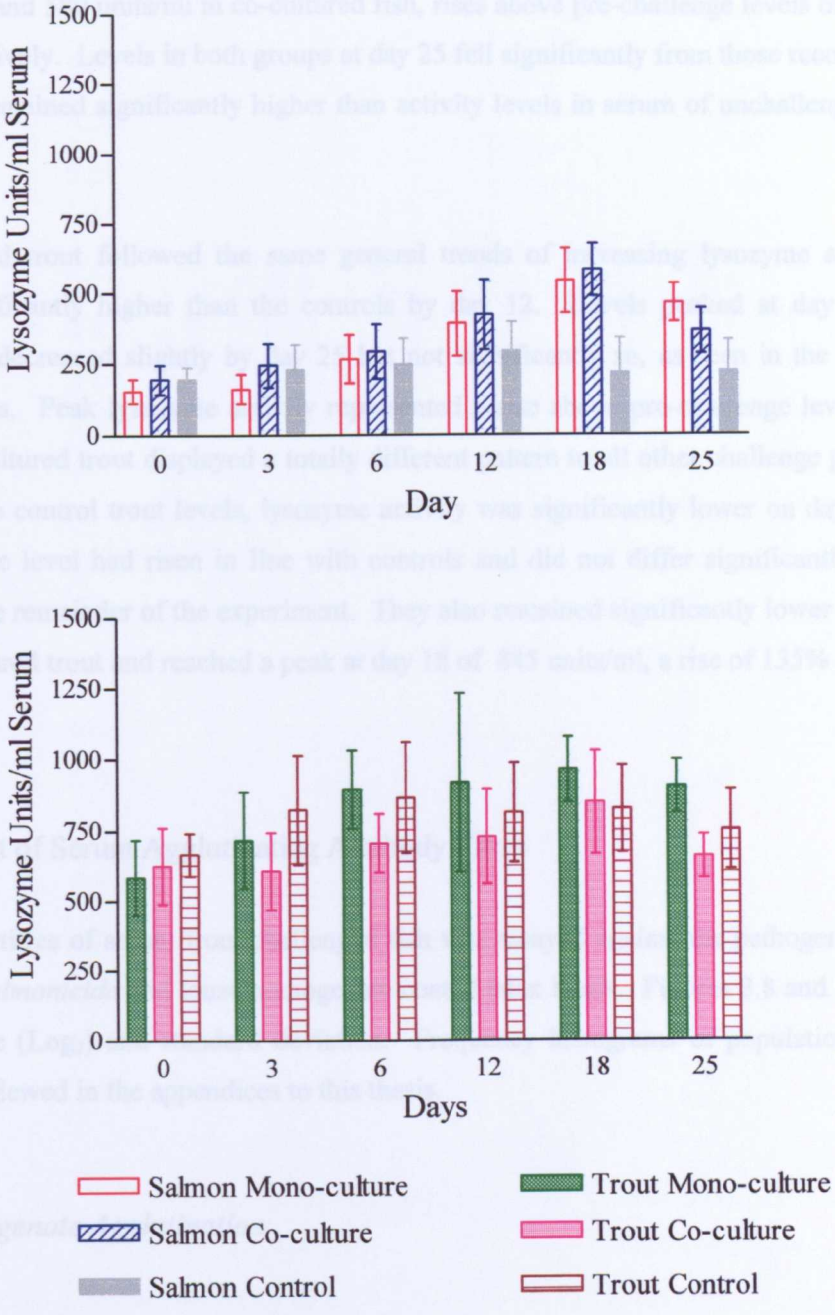


Figure 3.7

Mean number (± standard deviation) of lysozyme activity units in the serum of Atlantic salmon and rainbow trout following experimental infection with *L. salmonis*. Day 0 represents data from samples collected from the population prior to challenge (n=8). At all subsequent time points n=20 fish per group.

fish were 421 units/ml, a rise of 251% from pre-challenge levels, and 390 units/ml in mono-cultured salmon, a rise of 215%. Activity levels peaked at day 18 at 541 units/ml in mono-cultured fish and 580 units/ml in co-cultured fish, rises above pre-challenge levels of 349% and 297% respectively. Levels in both groups at day 25 fell significantly from those recorded at day 18 but still remained significantly higher than activity levels in serum of unchallenged control fish.

Mono-cultured trout followed the same general trends of increasing lysozyme activity and became significantly higher than the controls by day 12. Levels peaked at day 18 at 960 units/ml and decreased slightly by day 25 but not significantly so, as seen in the challenged salmon groups. Peak lysozyme activity represented a rise above pre-challenge levels of only 164%. Co-cultured trout displayed a totally different pattern to all other challenge groups. By comparison to control trout levels, lysozyme activity was significantly lower on days 3 and 6. By day 12 the level had risen in line with controls and did not differ significantly from the control for the remainder of the experiment. They also remained significantly lower than levels of mono-cultured trout and reached a peak at day 18 of 845 units/ml, a rise of 135% above base levels.

Measurement of Serum Agglutinating Antibody Titres

The antibody titres of serum from challenged fish was assayed against the pathogenic bacteria *Aeromonas salmonicida* and louse homogenate coated latex beads. Figures 3.8 and 3.9 present the mean titre (Log_2) and standard deviation. Frequency histograms of population antibody titres can be viewed in the appendices to this thesis.

Louse Homogenate Agglutination

Large variations in titre exist within all groups, however, statistical analysis using one-way analysis of variance and paired two-sample t-test did allow identification of significant differences between groups. The significance of data from salmon groups may be questioned since titres recorded pre-challenge are significantly lower than all subsequent data collected during the challenge in all groups. However, data from the challenge period shows antibody titres in challenged salmon groups that are significantly higher than seen in control fish from day 12 onwards. Data from salmon maintained in mono-culture and in co-culture are not significantly different.

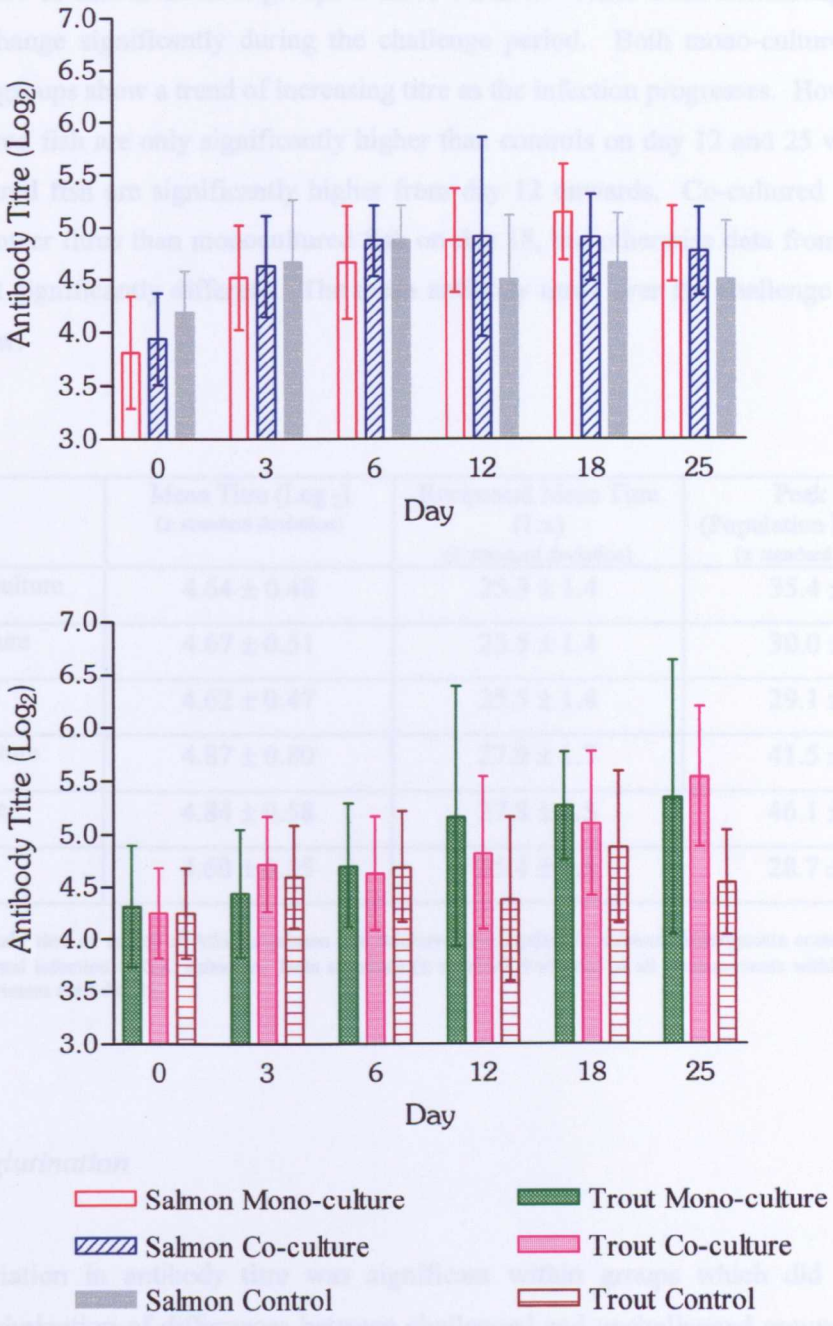


Figure 3.8

Mean (± standard deviation) agglutinating antibody titre (Log_2) of serum from Atlantic salmon and rainbow trout against latex beads coated with whole adult louse homogenate. Sera were collected from fish following experimental infection with *L. salmonis*. Day 0 represents data from samples collected from the population prior to challenge (n=8). At all subsequent time points n=20 fish per group.

The significance of data from trout groups is more variable. Titres from unchallenged control fish do not change significantly during the challenge period. Both mono-cultured and co-cultured trout groups show a trend of increasing titre as the infection progresses. However, data from co-cultured fish are only significantly higher than controls on day 12 and 25 whilst titres of mono-cultured fish are significantly higher from day 12 onwards. Co-cultured trout show significantly lower titres than monocultured fish on day 18, but otherwise data from these two groups are not significantly different. The mean antibody titres over the challenge period are tabulated below.

	Mean Titre (Log_2) (± standard deviation)	Reciprocal Mean Titre (1:x) (± standard deviation)	Peak Titre (Population Mean) (1:x) (± standard deviation)
Salmon Mono-culture	4.64 ± 0.48	25.3 ± 1.4	35.4 ± 1.4
Salmon Co-culture	4.67 ± 0.51	25.5 ± 1.4	30.0 ± 1.9
Salmon Control	4.62 ± 0.47	25.5 ± 1.4	29.1 ± 1.3
Trout Mono-culture	4.87 ± 0.80	27.9 ± 1.7	41.5 ± 2.4
Trout Co-culture	4.84 ± 0.58	27.8 ± 1.5	46.1 ± 1.6
Trout Control	4.60 ± 0.55	25.4 ± 1.5	28.7 ± 1.6

Table 2.1
Agglutinating antibody titre of serum of Atlantic salmon and rainbow trout against *L. salmonis* homogenate coated latex beads following experimental infection with *L. salmonis*. Data are mean (± standard deviation) of all measurements within groups from the start of the experiment until day 25.

Bacterial Agglutination

Individual variation in antibody titre was significant within groups which did not allow statistical discrimination of differences between challenged and unchallenged groups, between species, or between culture condition at any time point during the challenge. There was a general, although not significant tendency of higher titre in rainbow trout, however, the results in all cases indicate no significant changes in titre in any group as a result of sea lice infection. The population mean antibody titres are tabulated below.

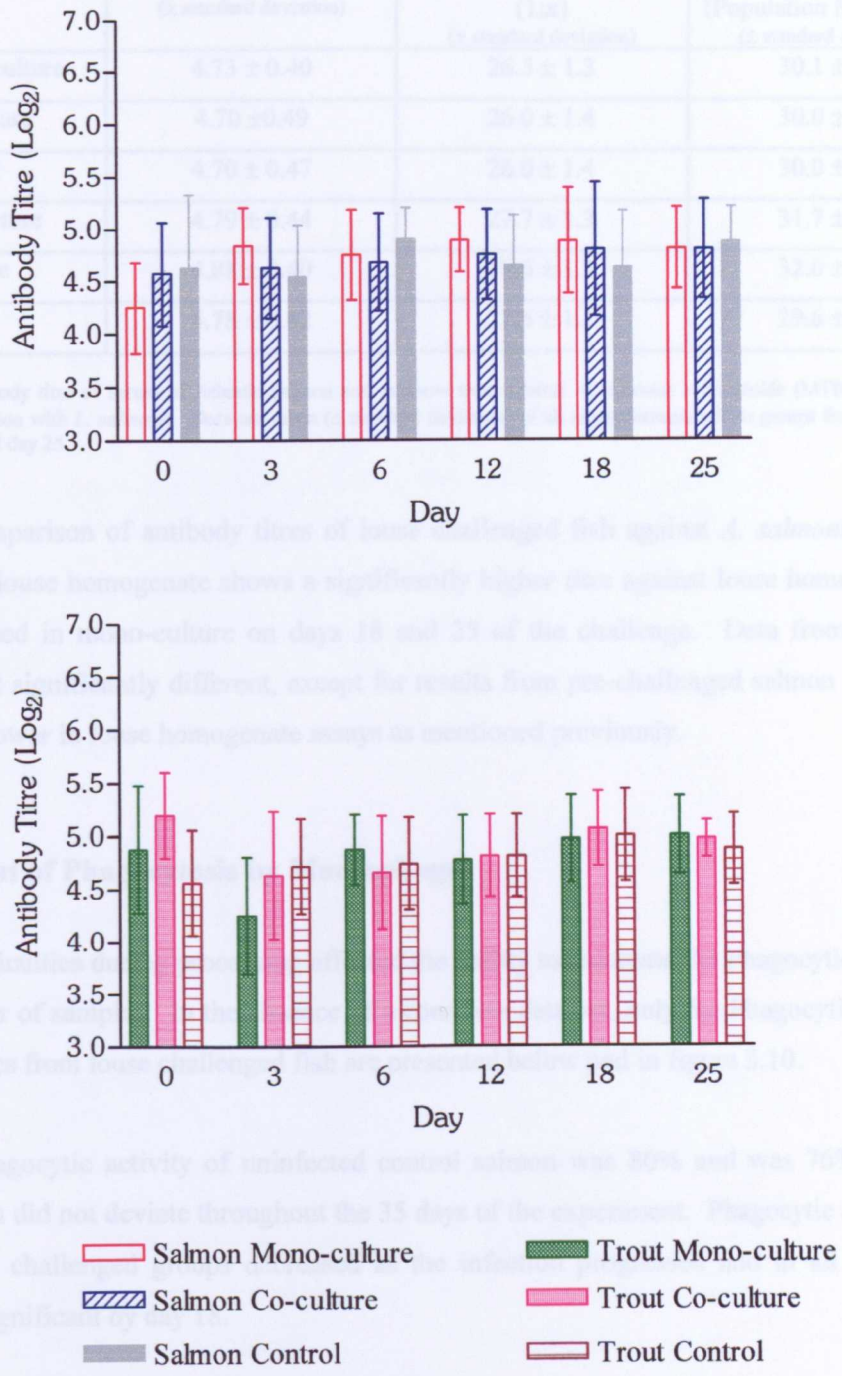


Figure 3.9

Mean (± standard deviation) agglutinating antibody titre (Log_2) of serum from Atlantic salmon and rainbow trout against *Aeromonas salmonicida* (MT004). Sera were collected from fish following experimental infection with *L. salmonis*. Day 0 represents data from samples collected from the population prior to challenge (n=8). At all subsequent time points n=20 fish per group.

	Mean Titre (Log_2) (± standard deviation)	Reciprocal Mean Titre (1:x) (± standard deviation)	Peak Titre (Population Mean) (1:x) (± standard deviation)
Salmon Mono-culture	4.73 ± 0.40	26.5 ± 1.3	30.1 ± 1.2
Salmon Co-culture	4.70 ±0.49	26.0 ± 1.4	30.0 ± 1.5
Salmon Control	4.70 ± 0.47	26.0 ± 1.4	30.0 ± 1.2
Trout Mono-culture	4.79 ± 0.44	27.7 ± 1.3	31.7 ± 1.3
Trout Co-culture	4.88 ± 0.40	29.6 ± 1.3	32.0 ± 1.2
Trout Control	4.78 ± 0.42	27.5 ± 1.3	29.6 ± 1.2

Table 2.2
Agglutinating antibody titre of serum of Atlantic salmon and rainbow trout against *Aeromonas salmonicida* (MT004) following experimental infection with *L. salmonis*. Data are mean (± standard deviation) of all measurements within groups from the start of the experiment until day 25.

Statistical comparison of antibody titres of louse challenged fish against *A. salmonicida* with those against louse homogenate shows a significantly higher titre against louse homogenate in trout maintained in mono-culture on days 18 and 25 of the challenge. Data from all other groups are not significantly different, except for results from pre-challenged salmon which are significantly lower in louse homogenate assays as mentioned previously.

Measurement of Phagocytosis by Macrophages

Technical difficulties during processing affected the ability to calculate the Phagocytic Index of a large number of samples. In the absence of a complete data set, only the Phagocytic Activity of macrophages from louse challenged fish are presented below and in figure 3.10.

The mean phagocytic activity of uninfected control salmon was 80% and was 76% in trout controls which did not deviate throughout the 35 days of the experiment. Phagocytic activity of cells from all challenged groups decreased as the infection progressed and in all cases the decline was significant by day 18.

Activity of cells from salmon and trout challenged in mono-culture did not differ significantly during the experiment and both fell to around 60% by day 35. The percentage of phagocytosing cells did however vary significantly between salmon and trout challenged in co-culture from day 18 onwards. Phagocytic activity of cells from salmon were significantly lower than recorded from trout. By day 35 the activity from co-cultured trout was 63% and was only 53% in co-cultured salmon. Activity of cells from co-cultured salmon were significantly lower than any

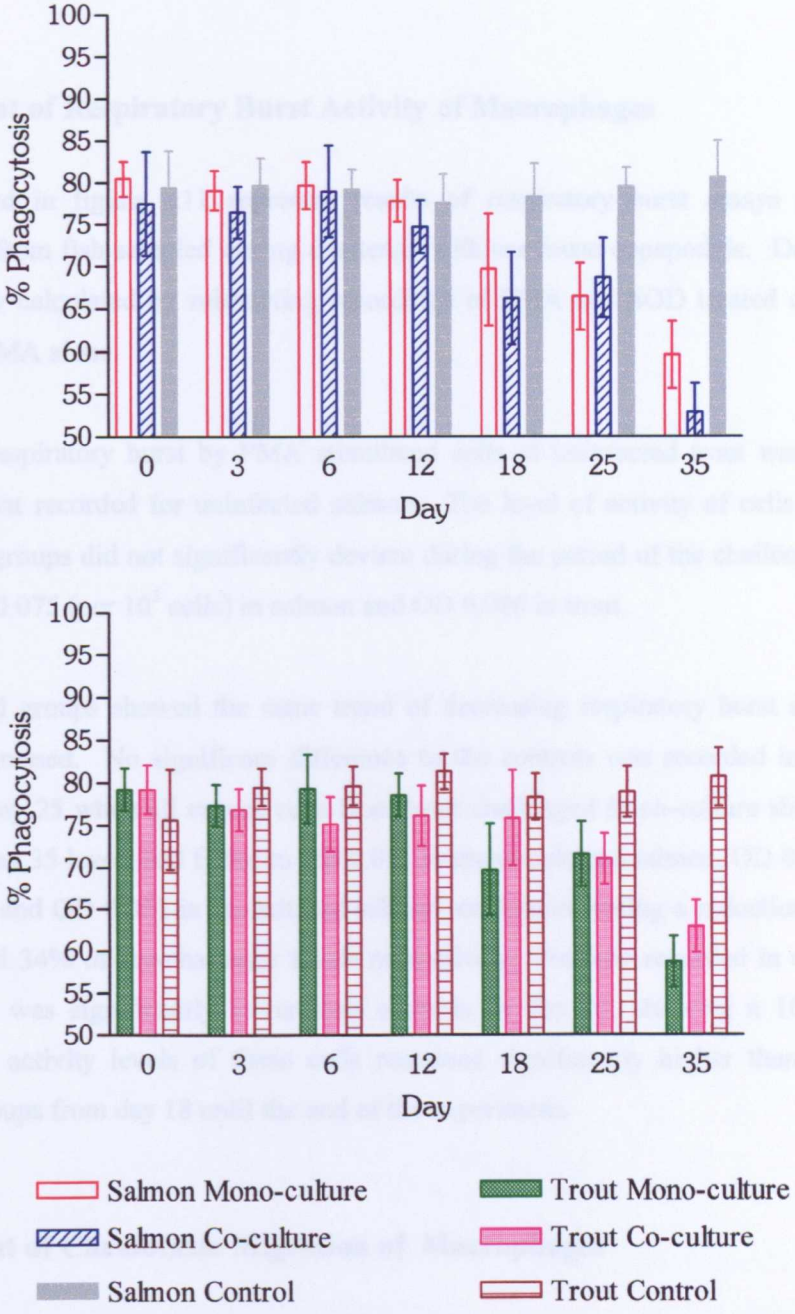


Figure 3.10

Mean (± standard deviation) percent phagocytosis of yeast by head kidney macrophages isolated from Atlantic salmon and rainbow trout following experimental infection with *L. salmonis*. Day 0 represents data from samples collected prior to challenge. At each time point n=6 fish per group.

other challenge group by day 35. The activity of cells from co-cultured trout was higher than all other groups at this time, but only significantly higher than co-cultured salmon.

Measurement of Respiratory Burst Activity of Macrophages

Data presented in figure 3.11 represent results of respiratory burst assays performed on macrophages from fish sampled during challenge with sea louse copepodids. Data are the net optical density calculated by subtracting recordings of PMA and SOD treated cells from cell treated with PMA alone.

Intracellular respiratory burst by PMA stimulated cells of uninfected trout was significantly higher than that recorded for uninfected salmon. The level of activity of cells from each of these control groups did not significantly deviate during the period of the challenge from mean values of OD 0.075 (per 10^5 cells) in salmon and OD 0.086 in trout.

All challenged groups showed the same trend of decreasing respiratory burst activity as the infection progressed. No significant difference to the controls was recorded in any of these groups until day 25 when all except cells from trout challenged in co-culture showed reduced activity. By day 35 levels had fallen to OD 0.051 in mono-cultured salmon, OD 0.061 in mono-cultured trout and OD 0.051 in co-cultured salmon, each representing a reduction inactivity of 32%, 29% and 34% of pre-challenge levels respectively. Activity recorded in cells from co-cultured trout was significantly lower than controls by day 35, showing a 10% reduction. However, the activity levels of these cells remained significantly higher than in the other challenged groups from day 18 until the end of the experiment.

Measurement of Chemotaxic Migration of Macrophages

The number of head kidney macrophages, isolated from challenged fish, that migrated towards 5% salmon serum, a known chemoattractant, was recorded from 3 fish from each species per tank at each time point over the 35 days of this experiment. Data are presented in figure 3.12.

The ability of macrophages from uninfected fish, both salmon and trout, to move towards the attractant did not deviate significantly from the average number of 175 cells per field of view at any time during the trial. All challenged fish demonstrated a reduced chemotaxic ability as the

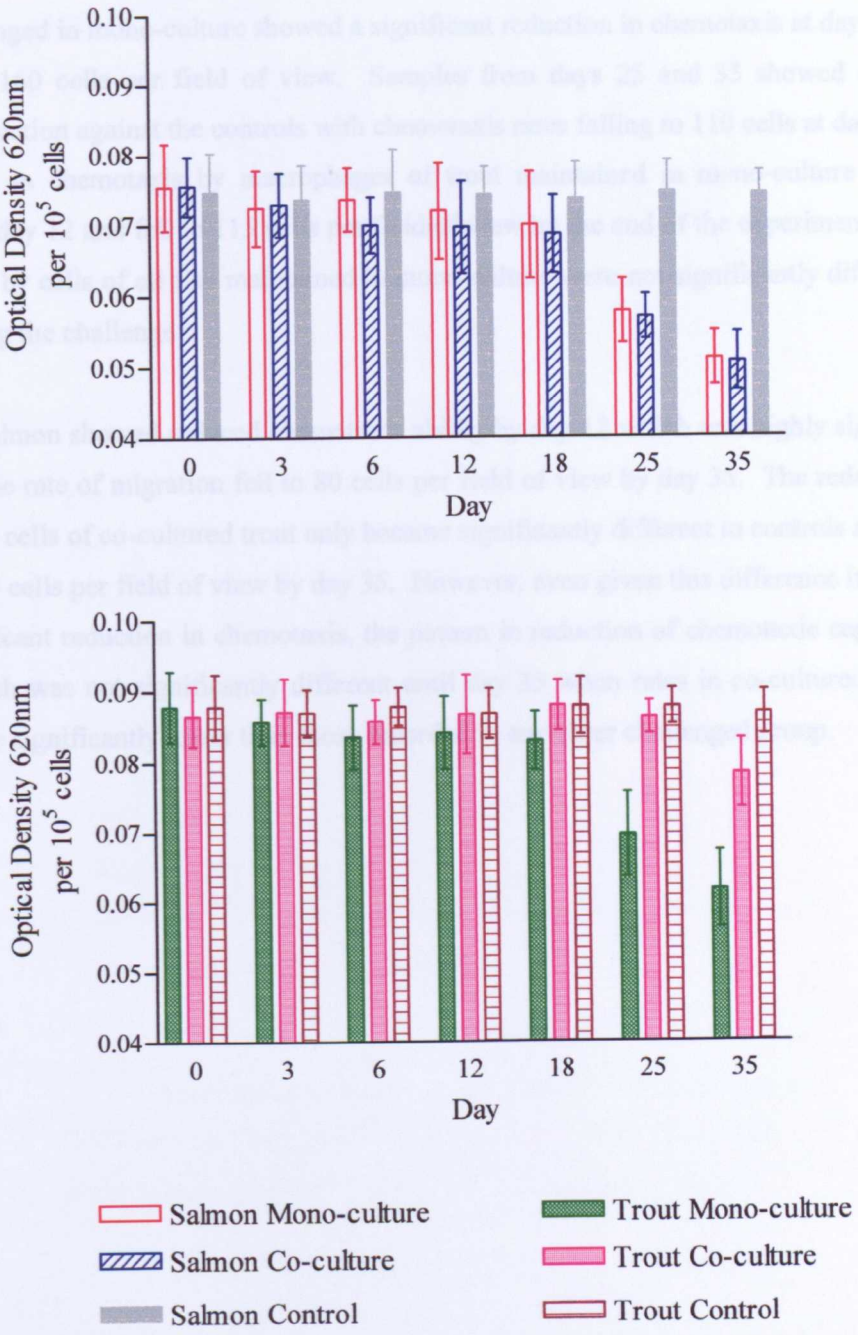


Figure 3.11

Mean (± standard deviation) respiratory burst activity by head kidney macrophages isolated from Atlantic salmon and rainbow trout following experimental infection with *L. salmonis*. Data are net optical density of measurements of PMA stimulated cells and of PMA and SOD treated cells. Day 0 represents data from samples collected for the population prior to challenge. At each time point n=6 fish per group.

louse infection progressed. However, there are differences between each group as to the extent of the depression in chemotaxis and the time post infection that the depression began to occur.

Salmon challenged in mono-culture showed a significant reduction in chemotaxis at day 18 with a decline to 160 cells per field of view. Samples from days 25 and 35 showed a highly significant reduction against the controls with chemotaxis rates falling to 110 cells at day 35. The reduction in chemotaxis by macrophages of trout maintained in mono-culture became significant by day 12 and fell to 115 cells per field of view by the end of the experiment. Rates of chemotaxis by cells of all fish maintained in mono-culture were not significantly different at any time during the challenge.

Co-cultured salmon showed reduced chemotaxic ability by day 12 which was highly significant by day 25. The rate of migration fell to 80 cells per field of view by day 35. The reduction in chemotaxis by cells of co-cultured trout only became significantly different to controls at day 25 and fell to 110 cells per field of view by day 35. However, even given this difference in the on-set of a significant reduction in chemotaxis, the pattern in reduction of chemotaxic capacity of co-cultured fish was not significantly different until day 35 when rates in co-cultured salmon (80 cells) were significantly lower than those recorded in any other challenged group.

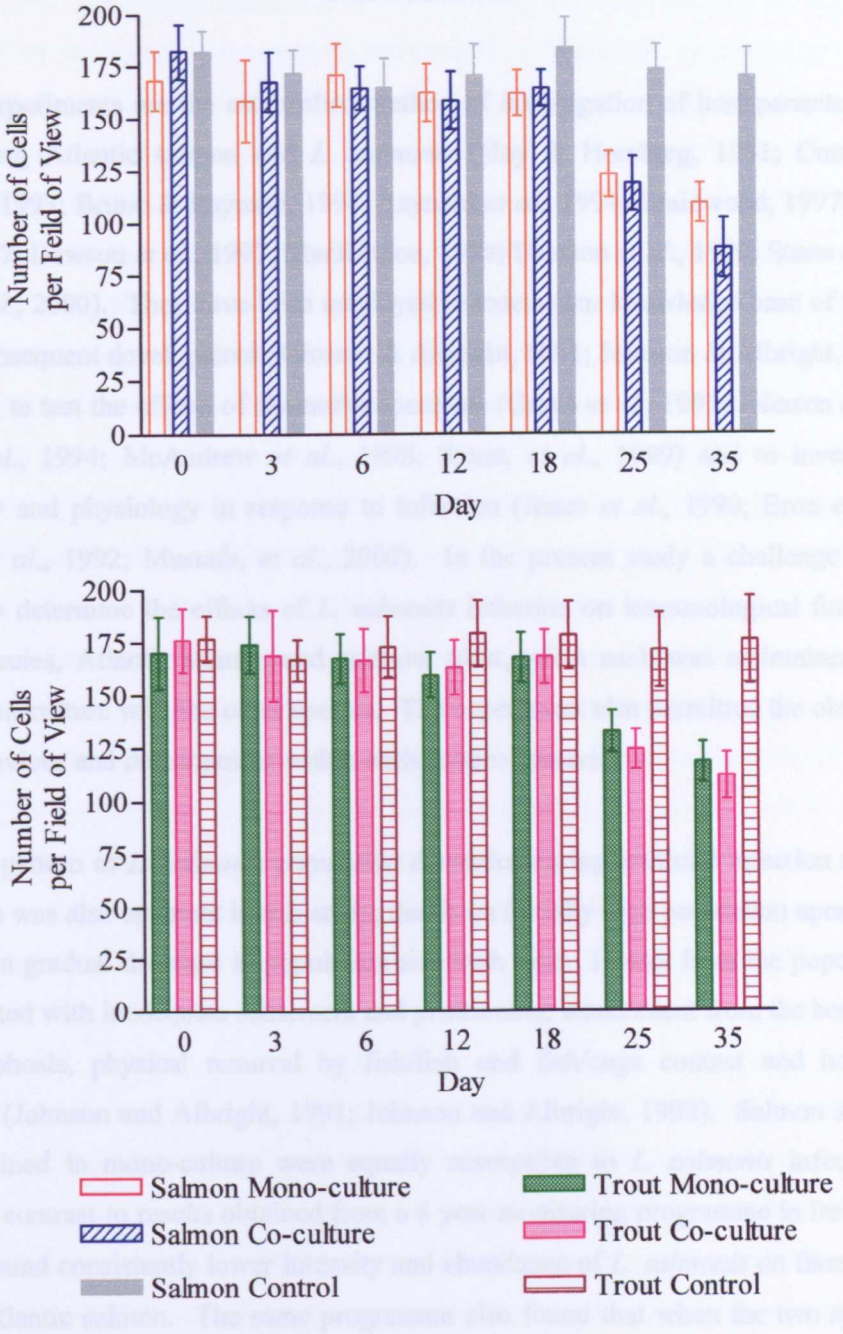


Figure 3.12

Mean (± standard deviation) rate of chemotaxis by head kidney macrophages isolated from Atlantic salmon and rainbow trout following experimental infection with L. salmonis. Data are the net of cells migrating towards a chemoattractant and cells migrating randomly. Day 0 represents data from samples collected from the population prior to challenge. At each time point n=6 fish per group.

DISCUSSION

Challenge experiments are the established method of investigation of host-parasite interaction when studying Atlantic salmon and *L. salmonis* (Hoy & Horsberg, 1991; Costello, 1993; Mackinnon, 1993; Bruno & Raynard, 1994; Raynard *et al.*, 1994; Braidwood, 1997; Brandal & Egidius, 1977; Dawson *et al.*, 1997; MacKinnon, 1998; Dawson *et al.*, 1999; Stone *et al.*, 1999; Mustafa *et al.*, 2000). They have been employed to extend our knowledge base of parasite life cycle and subsequent development (Johnson & Albright, 1991; Johnson & Albright, 1992; Bron *et al.*, 1993), to test the effects of chemotherapeutants (Grave *et al.*, 1991; Johnson *et al.*, 1993; Jenkins *et al.*, 1994; McAndrew *et al.*, 1998; Stone, *et al.*, 1999) and to investigate host pathobiology and physiology in response to infection (Jones *et al.*, 1990; Bron *et al.*, 1991; Jonsdottir *et al.*, 1992; Mustafa, *et al.*, 2000). In the present study a challenge model was developed to determine the effects of *L. salmonis* infection on immunological functioning of two host species, Atlantic salmon and rainbow trout, when each was maintained in mono-culture and co-culture with the other species. The experiment also permitted the observation of parasite behaviour and development within each culture scenario.

The general pattern of *L. salmonis* population dynamics during artificial infection recorded by other authors was also apparent in this study; that is an initially high population upon settlement followed by a gradual decrease in population size with time. Losses from the population have been associated with inadequate settlement and positioning, detachment from the host and death at metamorphosis, physical removal by fish/fish and fish/cage contact and host immune mechanisms (Johnson and Albright, 1991; Johnson and Albright, 1992). Salmon and rainbow trout maintained in mono-culture were equally susceptible to *L. salmonis* infection. This finding is in contrast to results obtained from a 6 year monitoring programme in Ireland (ICES, 1997) that found consistently lower intensity and abundance of *L. salmonis* on farmed rainbow trout than Atlantic salmon. The same programme also found that when the two species were reared together rainbow trout had lower louse burdens. These findings are echoed in this project where co-cultured salmon suffered significantly higher parasite burden than rainbow trout maintained in the same tanks. These differences were apparent from the first sampling point but were greatest at day 12 post infection when the louse population was 35% lower on rainbow trout. Following initial settlement and metamorphosis to chalimus stages, the rate of development of larvae on either salmon or rainbow trout hosts, whether maintained in mono- or co-culture, was not significantly different. Both species are obviously suitable hosts for *L. salmonis*, however the increased abundance of lice on co-cultured salmon strongly indicates a

'preference' for this species. The population dynamics recorded from this infection suggests a scenario whereby copepodid settlement on the two species may initially be equal but that detachment from less optimal hosts and re-settlement on alternatives, suggested by both Kabata (1979) and Bron *et al.* (1991) as a larval host finding strategy, occurred in the window between settlement and metamorphosis. Those copepodids that settled on rainbow trout that were outside this theoretical developmental window were unable to relocate but were non the less still able to complete their life cycle successfully on this host. Similar findings were made by Dawson *et al.* (1997) who reported that sea trout maintained in co-culture with Atlantic salmon suffered increased louse burdens following experimental infection, but that the rate of development of lice on each species was not impaired. Dawson *et al.* (1997) also suggested that the differences in copepodid settlement were a result of either differential exposure to copepodids during challenge, or enhanced immunological clearing of larvae by Atlantic salmon, but failed to establish this experimentally. Similar arguments were made in an ICES report (ICES 1997), which attributed lowered sea lice burdens on Atlantic salmon to the fish's more rapid and greater humoral responses. Both publications suggest that these are adaptations of a natural host species to allow balanced parasitism. However, such interactive balances may not exist within the artificial setting of aquarium tank trials and to conclude that Atlantic salmon are the natural and definitive host of *L. salmonis* based on this evidence is not entirely appropriate. However, the initial discussion of results from this present study may provide supporting evidence of this claim, although these experiments were conducted using rainbow trout and not sea trout, which is the primary species of contention in the debate on the effects of salmon farming on wild fish populations.

Measurements of serum immune parameters provided more incisive information on the comparative susceptibility of these two species and gives further scope for argument on the nature of the natural host. However, the investigation of long term and specific immunology of infected fish was not possible since the intensity of the louse infection forced the duration of the challenge to be shortened and a complete understanding of the immunological effects was not possible. It had been the intention to test the observations of Grayson *et al.* (1991) who found that serum antibody response to louse antigens measured from naturally infected salmon was very low. In the same experiment infected rainbow trout produced higher titres but these were also very low. In the present study specific antibody responses to adult louse antigens and to *Aeromonas salmonicida* antigens were also very low. During the course of the infection the serum antibody responses of both species did not change significantly. However, in response to louse antigens, slight increases were measured but still produced antibody titres of only 1:35 in salmon and 1:46 in trout. The aim of these measurements was to determine long-term antibody

production within the culture environments provided. Whilst a period of study longer period of study may have indicated more dramatic changes in responses, the wide variation in titres between individuals might not have allowed discrimination of any significant changes, especially since the expectation from previous studies is that antibody titres would remain low (Grayson *et al.*, 1991; Raynard, *et al.*, 1994)

Several authors have examined aspects of the haematology of *L. salmonis* infected fish, but no single citation exists that makes an extensive study of these parameters. In the most part measurements have been made of serological and haematological indicators of stress such as levels of glucose and cortisol (Mustafa *et al*, 1998, 2000) whilst Ruane *et al* (2000) also included measurement of packed cell volume (PCV), and erythrocyte and lymphocyte numbers. The present study made measurements of PCVs, total circulating blood cells, total leucocyte numbers and total serum protein concentration.

Salmon maintained in mono- and co-culture, as well as trout in mono-culture all showed significant changes in their PCVs. In each case the percentage of erythrocytes increased during the challenge, which was then followed by a rapid decline and severe anaemia. Trout in co-culture with Atlantic salmon did not show the same pattern, and PCVs did not differ significantly from those of unchallenged control fish. The measurement of total circulating erythrocytes and leucocytes shows an initial stable population followed by a rapid decline in both parameters in infected salmon and rainbow trout. Results of serum protein levels show gradually increasing concentrations in infected fish, followed by a rapid decline at the end of the experiment. Each of these observations, which were not identified by Ruane *et al.* (2000) in their study, indicates a physiological effect on haematology rather than an immunological effect. Changes in blood parameters during parasite infection may be interpreted as manifestations of either the host immune response to the infection, or of parasite induced modulation of the host system. However, the lack of supporting evidence for either of these allows a third alternative to be suggested; that being the scenario that initial tissue damage caused by early louse stages results in low level and gradual loss of fluid as osmoregulation is compromised leading to the apparent concentration of blood elements. Continued parasite development and damage associated with pre-adult lice (found at approximately day 12 onwards) appears to result in more extensive water loss and bleeding from wounds which removes blood elements from circulation and turns the apparent haemoconcentration into significant haemodilution. The rates of concentration and dilution of blood factors between the species groups does not provide the supporting evidence for the claim of differential susceptibility to *L. salmonis* infection. Whilst mono-cultured trout and salmon appear equally

affected by the progression of the infection, co-cultured salmon are significantly more affected than any of the other groups. These observations can be explained by the abundance of lice infecting these fish at the start of the experiment and are not necessarily linked to the animals' ability to challenge the infection and prevent or impede these haematological effects.

The measurement of serum lysozyme activity showed initial increases in levels in challenged salmon groups to more than double the resting level. Mono-cultured trout also showed increased levels of lysozyme, but not of the magnitude seen in the salmon. Levels in co-cultured trout did not vary significantly until the end of the experiment. The increase in activity levels is followed by a rapid decline at the end of the experiment which may link these measurements to the physiological processes of haemoconcentration and haemodilution discussed above, and not to the immunological response of the infected fish. Changes in lysozyme activity are known to be greatly influenced by stress and the class of stress chemical produced (Ruane, *et al.*, 2000). A reduction following stress has been seen in several fish species (Mock & Peters, 1990; Yin *et al.*, 1995; Hutchinson & Manning, 1996) whilst increases have also been reported (Mock and Peters, 1990; Demers & Bayne, 1997; Rotlant *et al.*, 1997). Both Ruane *et al.* (2000) and O'Flaherty *et al.* (1999) report elevated lysozyme activity in *L. salmonis* infected rainbow trout and sea trout respectively, which they attribute to a defence response from the fish. They both also report a gradual decline in activity as the infection progresses, in line with the findings of the present study, which they explain as a chronic stress response. The nature and the mechanics of the stressors' effects, either on reducing lysozyme production and activity, or its localisation, since relocation of blood elements to peripheral tissues such as skin and gills is a typical response to some stressors (Wendelaar Bonga, 1997), is not clear. However, it is also likely that the extensive damage to the integument caused by the infection in this study had some additional physical effects on the dilution and removal from the fish of blood elements, including lysozyme.

The measurement of the cellular immune functioning of each group aimed not only to quantify the effect of *L. salmonis* infection on the parameter being studied, but also to correlate the effect to the overall susceptibility of each group to infection. This study measured phagocytosis of yeast, macrophage chemotaxis and intra-cellular respiratory burst. Each test used is an accepted *in vitro* indicator of macrophage functioning (Paulnock, 2000) and they have been used in the study of *L. salmonis* infections of Atlantic salmon by Mustafa *et al.* (1998, 2000). In the latter study (Mustafa *et al*, 2000) the authors found that respiratory burst and phagocytic activity was significantly decreased by day 21 post infection with sea lice. In the present study the phagocytic activity of cells isolated from infected fish, both Atlantic salmon and rainbow trout,

declined significantly by day 18 post infection, the respiratory burst activity declined significantly by day 25, as did the rate of cell migration, measured by the chemotaxis assay. The infection therefore resulted in significant depression of the cellular immune system of both Atlantic salmon and rainbow trout, but the nature of the depression is unclear. It may be argued that secreted substances from attached lice caused systemic depressive effects as is seen in many crustacean parasite infections of terrestrially farmed animals (Wikel *et al.*, 1994; Wikel *et al.*, 1996), alternatively the physiological changes in haematology that we attribute to the failure of osmoregulation may also be manifest in the abnormal functioning of lymphoid tissues. Mustafa *et al.* (2000) suggest a third explanation, one for which they have experimental evidence. In addition to measuring cellular immune parameters they also measured serum cortisol and glucose levels, both indicators of stress. In their work they found levels of these substances to be significantly increased at day 21 post challenge, which correlates to the timing of decreased cellular immune function. Chronic stress and the persistence of circulating stress chemicals results in maladaptation and the modulation of normal physiology and can induce a generalised and long-term immune suppression (Schreck, 1996). The point at which a stressor moves from being acute to chronic is debatable, but 18 or 25 days of parasite infection, the incidence of significant decline in cellular immune parameters in this study, would seem to qualify as chronic stress and may explain the immunosuppression recorded. In another study, this time in rainbow trout, Narnaware *et al.* (1994) showed significant depression of phagocytosis following acute stress, which they concluded, was caused by the action of catecholamines. The lack of apparent immunomodulation in either this study or the work by Mustafa *et al.* (2000) in the period at the start of the infection that might be considered 'acute phase' is likely to be as a result of the low impact and tissue damage of the early larval lice. In both studies the appearance of pre-adult lice coincides with the development of significant epidermal injury, and in this present study is also characterised by haemodilution. These studies indicate that sea lice infections induce a stress response and a related immune suppression in host fish which is greatest when lice are mobile and larger and able to cause most damage. The increased impact of the later stages of louse development has also been noted by numerous authors (Pike, 1989; Mackinnon, 1993; Bjorn & Finstad, 1997).

The experimental evidence provided in this chapter demonstrates that Atlantic salmon and rainbow trout are equally susceptible to infection by *L. salmonis* and that their physiological and/or immunological responses to the infection do not differ significantly. However, increased lysozyme activity in infected salmon groups may be indicative of an aggressive response to the attached lice, which is not as marked in the trout groups. In mixed populations it appears that *L. salmonis* preferentially settle on Atlantic salmon but that individuals that do settle on rainbow

trout suffer no developmental or survival detriment. However, in this environment rainbow trout appear to demonstrate significantly reduced effects of the infection, whilst Atlantic salmon suffer effects significantly above those seen in salmon maintained in mono-culture. These effects are obviously a result of the increased louse population on salmon within mixed species environments and may explain the observations that salmon are comparatively more susceptible to louse infection when they are cultured together at mixed farm sites (ICES, 1997). The physiological and immunological parameters measured here do not provide evidence to explain the differential settlement of larvae in the co-culture situation and so cannot contribute to the debate surrounding the identification of the 'natural' host of *L. salmonis*. Whilst *L. salmonis* seem to show an apparent preference for Atlantic salmon over rainbow trout in tank trials, and from some field evidence (ICES, 1997), further experimental evidence is required to draw firm conclusions. These experiments should include sea trout as an additional species and be as extensive as is practical to reduce the possibility of tank effects and differential exposure to copepodids during the infection to finally draw a line under this contentious issue.

THE DEVELOPMENT OF A *L. SALMONIS* *IN VITRO* CULTURE ENVIRONMENT

Chapter 4

INTRODUCTION

THE DEVELOPMENT OF AN ORGANOTYPIC FISH SKIN MODEL

In vitro models of animal systems exist at all levels of complexity from monolayer culture of single cell types, explanted whole tissues, organotypic culture of multiple cell types to artificially designed and engineered tissue substitutes. Each system has particular benefits commensurate with its application whether that be chemical testing, disease diagnosis, clinical procedures or fundamental biological research.

Cell culture systems and model development in the veterinary field tend to 'lag' behind the technologies of human medical research (Freshney, 1998). However, these methods and technologies are more readily adopted by those working with higher vertebrates, specifically mammals, owing to the metabolic, anatomical and physiological similarities with the human system. Those working with lower vertebrates have not manipulated these technologies for application in the research of their own species, at least not extensively. The significant differences between human and lower vertebrate biology would not allow direct transfer of this technology, without first completing extensive, and expensive, preparatory research and development. However, as has been demonstrated by the applications and findings of such models in the study of human biology, complex cell culture systems could greatly benefit the understanding of the biology of all species.

Cell culture based *in vitro* investigation of teleost systems primarily takes the form of monolayer culture of single cell types (Wolf, 1979; Hightower & Renfro, 1988; Wolf & Mann, 1980), but the use of short term tissue explants is also common (Landreth & Agranoff, 1979; Anderson *et al.*, 1987; Grinblat *et al.*, 1999). Both systems have significant disadvantages which are accepted by those applying them in the absence of more suitable alternatives.

Explants offer short / medium term whole tissue models which maintain the structure, organisation and cell types and morphology of the tissue *in vivo*. However, long term maintenance is not possible and the tissue is in a state of physiological and biochemical degradation immediately following excision from the donor (Shaw, 1996; Freshney, 1998). The impact of these metabolic changes on the parameter being studied is not always known and results require cautious interpretation by the researcher.

Monolayer systems are the earliest and most extensively used cell culture models (Shaw, 1996; Freshney, 1998). However, when cells are isolated and propagated in such systems cell proliferation and not differentiation is encouraged. The resultant cell phenotype differs dramatically with increasing passage from the characteristics predominant in the original tissue by having significantly different morphology, biochemistry and function, or lack of.

Alternative methods for the *in vitro* culture of cell monolayers were developed in the inter-war years using extracellular material as the culture substrate (Shaw, 1996). Extracellular material such as collagen, laminin and fibronectin form the matrix of fibres and surfaces upon and around which cells grow to form tissue architecture. *In vivo*, cell-matrix interactions are known to be responsible for stimulation of types I and IV collagen synthesis by dermal fibroblasts (Lamme *et al.*, 1998), differentiation of the basement membrane and initiation of the keratinisation process within the skin (Shaw, 1996). In similar ways the extracellular matrix of all organs plays an integral part in defining cell types, structure and organisation of tissues. *In vitro*, monolayers of single cell types maintained on collagen extracellular matrix substrates (histotypic cell culture) have been seen to recover their differentiated function, cell shape and cell surface morphology (Shaw, 1996). Yamada *et al.* (1976) also report the recovery of cancer transformed cells to their normal phenotype when grown on collagen, whilst Jura *et al.* (1996) detail experiments where primary isolations of hepatocytes maintained their function and spontaneously re-organised into bile duct-like tissue when cultured with collagen.

Organotypic culture methods take the use of extracellular material a step further by using it as a framework, in some cases moulded to specific dimensions, on which is seeded mixed cell types from the tissue of study (Shaw, 1996; Freshney, 1998). In this configuration these cells are capable of reorganisation so as to structurally resemble the tissue *in vivo*, they express cell surface markers denoting specific cell types, and are capable of basic metabolic function such as transmission of nervous impulses (Michel *et al.*, 1999) and synthesis and secretion of hormones and extracellular enzymes (Douglas *et al.*, 1983; Herrera-Marschitz *et al.*, 2000). The development of organotypic systems of fish is not extensive. They have been used to recreate retinal tissue of the gold fish, *Carassius auratus*, with the ultimate aim of investigating optic neural responses and the regulation of rod cell division (Mack & Fernald, 1993), as well as being applied to the study of lateral line tissues of the marbled electric ray *Torpedo marmorata* (Richardson *et al.*, 1981). In this study, and in a remarkably similar study of another electric fish, *Apteronotus leptorhynchus*, the ghost knifefish, (Kotecha *et al.*, 1997), homogenised tissues were reconstituted onto collagen substrates which resulted in tissue-like organisation,

identification of specific cell types and visualisation of anatomical boundaries between functional regions of the tissue. Typically these cultures remained viable for only up to 27 days but allowed the isolation of electrosensory cells and electrophysiological analyses of neural membrane properties.

With regards to fish research this represents the limit of current cell culture technology. Mammalian, especially human cell culture, has advanced further to the point of the culturing of entire organs. The majority of the organs under study are many years away from complete success (Arnst & Carey, 1998) but significant advances have been made in the construction of artificial liver (Kaihara *et al.*, 2000), pancreas (Papas *et al.*, 1999), bone (Whang *et al.*, 1999) and heart (Hoerstrup *et al.*, 2000a, 2000b; Sodian *et al*, 2000a, 2000b). The development of artificial skin was the earliest and most successful attempt at organ culture (Phillips, 1993). Early work began in the 1970s with eventual success in the 1980s (Bell *et al.*, 1981a, 1981b; Bell *et al.*, 1983; Bell *et al.*, 1991a, 1991b; Parenteau *et al.*, 1991), and following extensive development and clinical trials it is now licensed for use as the first organ substitute.

The use of Living Skin Equivalents for parasitological research was first evaluated by Fusco *et al.* (1993) who tested its suitability in the study of host skin penetration by *Schistosoma mansoni* cercariae. They concluded that it could play a vital role in understanding both parasite mechanisms of penetration and host responses to it, but have not published any subsequent work on the topic in the scientific literature.

The development of a fish skin substitute, in theory, should involve the simple application of mammalian technologies to dissociated fish cells, since the physiological optimum environment, maintenance conditions and behaviour of isolated teleost cells is essentially the same as seen in mammalian cell culture, in fact most fish cell lines are maintained in media and with culture supplements designed primarily for human cell culture (Wolf, 1979; Freshney, 1998; Hightower and Renfro, 1988). However, functional tissue is not only formed by the association of correct cell type with extracellular matrix in the correct arrangement, it is also influenced by exogenous factors and conditions which shape the biochemical interactions between cells and leads to the required specialised adaptations. As well as cell-cell and cell-matrix interactions, fish skin *in vivo* interacts with its aqueous environment. As far as this author is aware there are no reports of cell culture models which operate within a discontinuous osmotic gradient, since regardless of whether the aqueous environment is fresh water (at <50 mOsm Kg^{-1}) or sea water (at 1000 mOsm Kg^{-1}) they are both significantly different to the osmolarity of cultured animal cells of approximately 300 mOsm Kg^{-1}. A fully functional fish

skin substitute would therefore be required to perform equally well in both hypotonic and hypertonic conditions. Since the aim of this project is to develop a cellular substrate for the maintenance of the marine parasite *L. salmonis* it is a required criterion that the substrate be tolerant of high osmolarity.

Several choices present themselves when approaching the problem of differential tonicity between the substrate and the requirements of the parasite:

1. The maintenance of the parasite at lowered osmolarity
2. The maintenance of the cells at increased osmolarity
3. The development of a physical barrier between the cells and the seawater media, but one that still permits parasite settlement
4. The development of fully functional fish skin containing specialised cell types and tissues, able to provide a physical barrier to seawater ingress and with osmoregulatory capabilities

Option four would not only represent a significant milestone in fish veterinary research but would also be a significant achievement within the field of tissue engineering since such a tissue is not yet a reality within mammalian tissue culture research (Arnst and Carey, 1998). The remaining 3 options provide more realistic choices for investigation.

The physiological tolerance ranges of adult sea lice have been much studied, and with regard to their tolerance to lowered salinity the study by Hahenekamp and Fyhn (1985) is possibly the most comprehensive. These authors studied the osmotic response of lice during transition from seawater to fresh water. They found significant differences in the survival rates of free swimming adult and attached adult lice. Attached lice were able to maintain their haemolymph osmolarity and chloride concentration and survived for at least a week in fresh water whilst free-swimming adults quickly hydrated and died within 8 hours of transfer. They hypothesised that attached individuals were able to maintain their salt balance through replenishment from the mucus and body fluids/tissues of the host, but that they did eventually succumb to hydration. Finstad *et al.* (1995) report that sea lice naturally infecting Arctic char, *Salvelinus alpinus*, were able to survive for up to 3 weeks in fresh water, however, they do not discriminate between the developmental stages of the louse and so provide no evidence of the osmotic tolerance of *L. salmonis* larvae.

Manipulation of the media osmolarity of cultured cells is often used to measure stress responses in cells following both acute and chronic exposure (Shaw, 1996). Medium is commonly

modified by the addition/subtraction of salts such as NaCl, and complex carbohydrates such as xylose, sorbitol and mannitol (Freshney, 1998). Such techniques have been used to study the responses of cultured fish cells. Hashimoto *et al.* (1999) found that they were able to adapt EPC cells to increased osmolarity, up to 600 mOsm Kg^{-1}. Cell death was a common feature of the cultures during the acclimation process but fully adapted cells were able to proliferate and form confluent monolayers at the higher osmolarity. Furthermore, they found that the degree of DNA fragmentation of high osmolarity adapted cells was significantly lower than that in cells maintained at physiological osmolarity, indicative of the suppression of cell apoptosis, the main feature of which is DNA fragmentation. The significance of these findings are still under investigation. The effects of increased osmolarity on cells from marine fish were investigated by Tocher *et al.* (1994). In this study they did not acclimate the cells to increased osmolarity but instead measured the effects of an abrupt change on the cells. They found that with increasing osmolarity, from 300 mOsm Kg^{-1} to 500 mOsm Kg^{-1}, both the peak cell number and proliferation rates declined. They also measured changes in the lipid metabolism and lipid profile of turbot, *Scopthalmus maximus*, cells where the percentage of total polar lipid classes increased at higher osmolarity. No such changes were found in Atlantic salmon cells in the same experiments which may indicate their ability to maintain normal cell metabolism, biochemistry and functioning with broader ranges of environmental change that they are likely to encounter in their anadromous life cycle.

This chapter will investigate whether indeed salmon cells are tolerant of changes in the osmolarity of their culture environment, as well as the extent to which *L. salmonis* larvae are tolerant of lowered salinities. It will also examine how the use of organotypic tissue culture techniques can be used to organise salmon cells into a skin-like structure capable of providing a physical barrier to seawater ingress into the culture system; option 3 from the choices listed above. There exists no known precedent for the application of these methods and materials in exactly this way and so the developmental stages of this aspect of the project will be described thoroughly.

MATERIALS AND METHODS

Chapter 4 will present the results of experiments aimed at determining the survival of *L. salmonis* larvae at sub-optimum levels of osmolarity (section 2.16). It will then detail a series of experiments whereby cultured Atlantic salmon cells are adapted to maintenance in hyperosmotic media (section 2.17) all in an attempt to modify the physiological optimum tonicity of these 2 study subjects to allow their co-culture within a single system.

Further experiments will show the developmental progression of a cell culture based substrate constructed with the specific aim of being a suitable surface for the settlement of *L. salmonis* larvae. Preliminary experiments will combine established Atlantic salmon cell lines with purified bovine collagen and monitor the effects on cell growth and organisation in both standard culture media (section 2.21) and full strength seawater (section 2.22). Changes in the construction and configuration of the substrate, and the resulting effects on cell survival will be examined (section 2.23 and 2.24).

This chapter will also detail the development of a salmon skin organotypic model (section 2.25) constructed using primary cell isolations from Atlantic salmon epidermis (2.25.1). The cell survival, tissue morphology (section 2.25.3) and differentiated functioning of cells within the model (section 2.26) will also be investigated.

RESULTS

Measurement of Survival of Copepodids Maintained in Sub-optimum Conditions

The survival of copepodids incubated with seawater diluted by the addition of fresh water is plotted in figure 4.1 The survival of animals maintained in seawater with osmolarities of 900 and 800 mOsmol Kg^{-1} did not vary significantly from that of larval lice in full strength seawater (1000 mOsmol Kg^{-1}). Survival of animals incubated in seawater at 700 mOsmol Kg^{-1} and below was significantly lower than at 1000 mOsmol Kg^{-1}. The duration of survival of 50% of each population maintained in osmotically modified seawater is tabulated below.

Treatment Group (mOsmol Kg^{-1})	50% Survival Period (days)
1000	8.27
900	8.15
800	7.12
700	5.22
600	4.0
500	4.0
400	1.85
300	0.9

The survival time of copepodids incubated with standard tissue culture media, also measured during this experiment, was less than 1 day. In an additional experiment to investigate this result, 10 copepodids were individually added to a volume of each culture media and their survival time recorded. In this experiment, media were also included that lacked phenol red (present in standard culture media formulations), and which were modified to 1000 mOsmol.Kg-1 by the addition of diluted seawater during the reconstitution of powdered media. These data are displayed in figure 4.2.

In all media tested, copepodid survival was less than 8 minutes which makes them unsuitable for use in an *in vitro* system for the culture of *L. salmonis*. Significant variation in survival

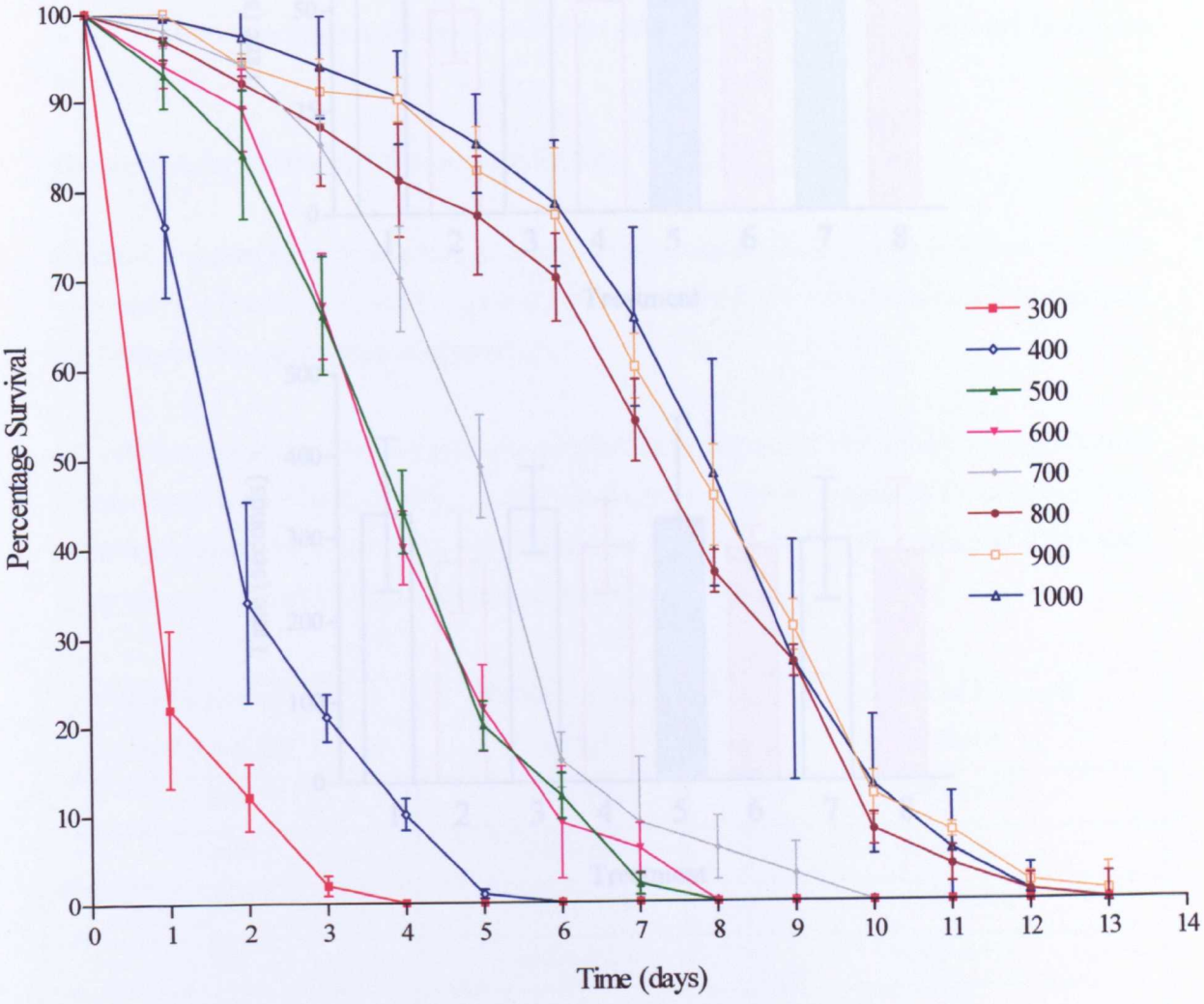


Figure 4.1

Mean percent survival (± standard deviation) of copepodids of *L. salmonis* maintained in seawater diluted by the addition of fresh water to osmolarities in the range 100 to 1000 mOsmol kg^{-1}. At each time point n=24.

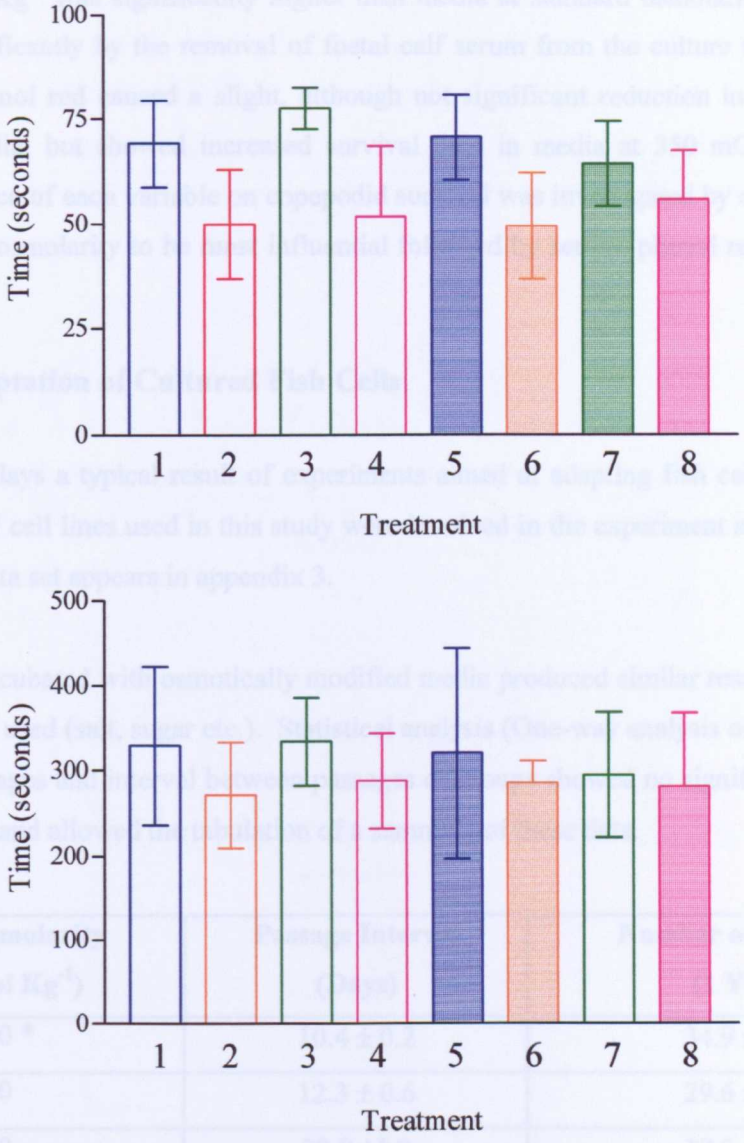


Figure 4.2

Mean (± standard deviation) survival time of copepodids of *L. salmonis* incubated with standard and modified tissue culture media. The uppermost graph displays data from media at standard osmolarity (350 mOsmol Kg^{-1}) and the lower graph displays data from media modified to 1000 mOsmol Kg^{-1} by the addition of diluted seawater to powdered media preparations. n=10

1= L-15, no serum; 2= L-15, 10% serum; 3= L-15, no serum, no phenol red; 4= L-15, 10% serum, no phenol red; 5= MEM, no serum; 6= MEM, 10% serum; 7= MEM, no serum, no phenol red; 8= MEM, 10% serum, no phenol red.

times between each media were however recorded. Survival in tissue culture media modified to 1000 mOsmol.Kg^{-1} was significantly higher than media at standard osmolarity. Survival was increased significantly by the removal of foetal calf serum from the culture media, whilst the absence of phenol red caused a slight, although not significant reduction in survival in high osmolarity media, but showed increased survival time in media at 350 mOsmol.Kg^{-1}. The relative influence of each variable on copepodid survival was investigated by covariate analysis which showed osmolarity to be most influential followed by serum, phenol red and finally the medium type.

Osmotic Adaptation of Cultured Fish Cells

Figure 4.3 displays a typical result of experiments aimed at adapting fish cell lines to higher osmolarity. All cell lines used in this study were involved in the experiment and a summary of the complete data set appears in appendix 3.

All cell lines incubated with osmotically modified media produced similar results regardless of osmopoteniator used (salt, sugar etc.). Statistical analysis (One-way analysis of variance) of the number of passages and interval between passages of groups showed no significant differences in performance and allowed the tabulation of a summary of these data.

Media Osmolarity (mOsmol Kg^{-1})	Passage Interval (Days)	Number of Passages (1 Year)
350 *	10.4 ± 0.2	34.9 ± 0.7
500	12.3 ± 0.6	29.6 ± 1.3
600	20.8 ±1.0	17.5 ± 0.9
700	32.6 ± 1.4	10.0 ± 1.0
800	0	0

* (normal culture media)

The performance of all cells maintained at 500 mOsmol Kg^{-1} was significantly different to that of cells maintained in standard culture media. The difference in performance of cells at 600, 700 and 800 mOsmol Kg^{-1} to those in standard media was highly significant. These cells performed less well than cells at 350 and 500 mOsmol Kg^{-1} in that the recorded interval between passages was significantly higher, and consequently the total number of viable passages was significantly lower. Furthermore, cells maintained at 700 mOsmol Kg^{-1} had a limited adapted life span (approximately 10 passages) which often meant that cultures were non-viable before the end of

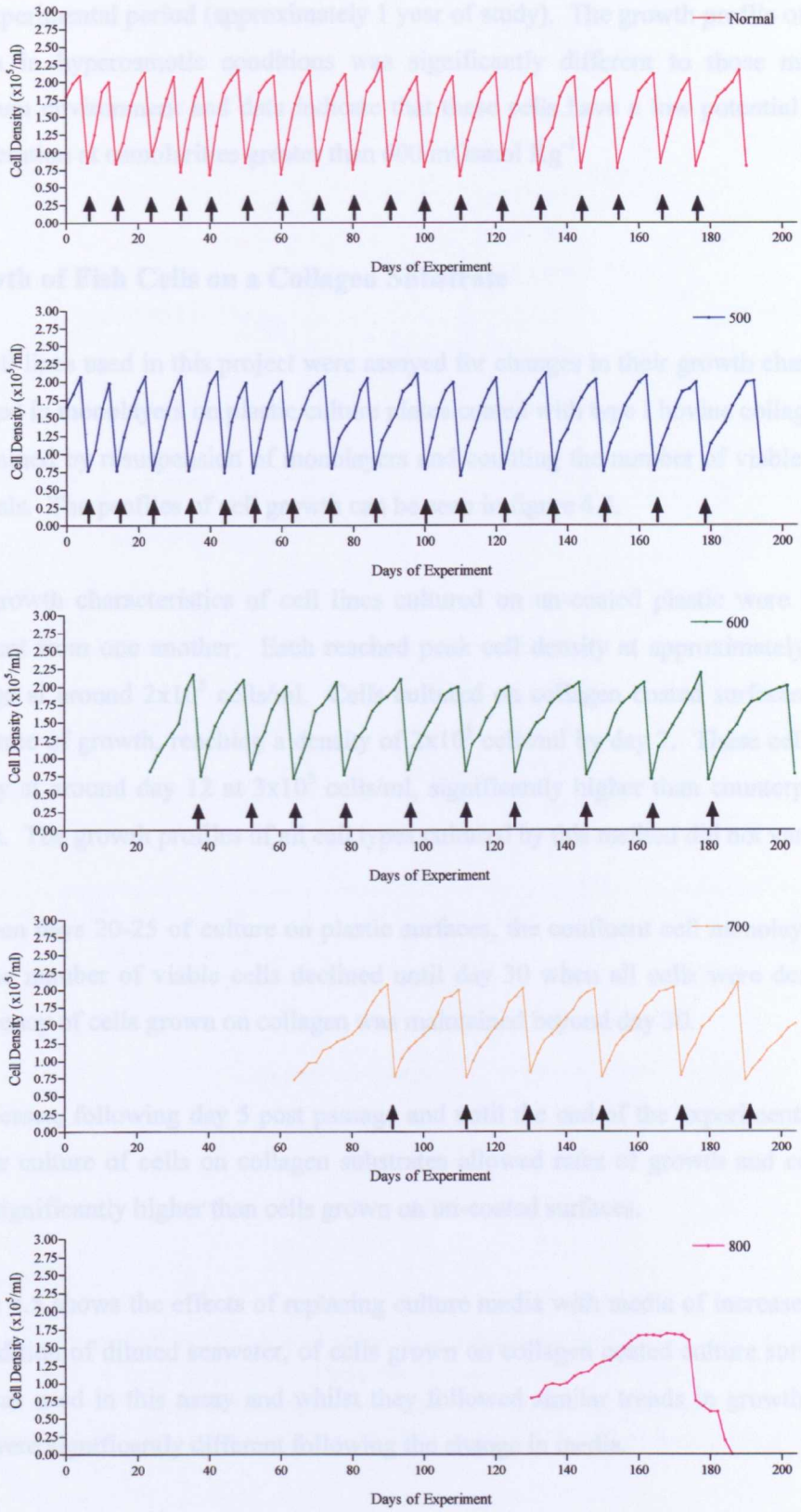


Figure 4.3

The passage interval and cell density achieved by cells maintained at increasing osmolarity. These data represent performance of AS-6 cells used in the adaptation experiments described in section 2.17. The arrows anotating each chart indicate the time of passage and the re-seeding of cells at approximately $1x10^5$/ml. Data for other cell lines used in this experiment were not significantly different to the data for AS-6 and are summarised in the appendices to this thesis.

the experimental period (approximately 1 year of study). The growth profile of cell populations grown in hyperosmotic conditions was significantly different to those maintained in the optimum environment and data indicate that these cells have a low potential for survival and proliferation at osmolarities greater than 600 mOsmol Kg^{-1}.

Growth of Fish Cells on a Collagen Substrate

All cell lines used in this project were assayed for changes in their growth characteristics when cultured in monolayers on plastic culture plates coated with type I bovine collagen. Growth was determined by resuspension of monolayers and counting the number of viable cells at 24 hour intervals. The profiles of cell growth can be seen in figure 4.4.

The growth characteristics of cell lines cultured on un-coated plastic were not significantly different from one another. Each reached peak cell density at approximately day 11-13 post passage at around $2x10^5$ cells/ml. Cells cultured on collagen coated surfaces showed a more rapid rate of growth, reaching a density of $2x10^5$ cells/ml by day 7. These cells achieved peak density at around day 12 at $3x10^5$ cells/ml, significantly higher than counterparts cultured on plastic. The growth profiles of all cell types cultured by this method did not vary significantly.

Between days 20-25 of culture on plastic surfaces, the confluent cell monolayers began to die and the number of viable cells declined until day 30 when all cells were dead. Conversely, confluency of cells grown on collagen was maintained beyond day 30.

In all cases, following day 5 post passage and until the end of the experimental period on day 30, the culture of cells on collagen substrates allowed rates of growth and cell densities that were significantly higher than cells grown on un-coated surfaces.

Figure 4.5 shows the effects of replacing culture media with media of increased osmolarity, by the addition of diluted seawater, of cells grown on collagen coated culture surfaces. Each cell line was used in this assay and whilst they followed similar trends in growth, the profiles of each were significantly different following the change in media.

Cell populations growing confluently at $3x10^5$ cells/ml experienced a rapid decline following the addition of a 25% seawater media. At 1 day post addition the cell population had fallen significantly from that of cells maintained in standard media. The decline in population plateaued at around day 15 (5 days post addition of the seawater media) at approximately

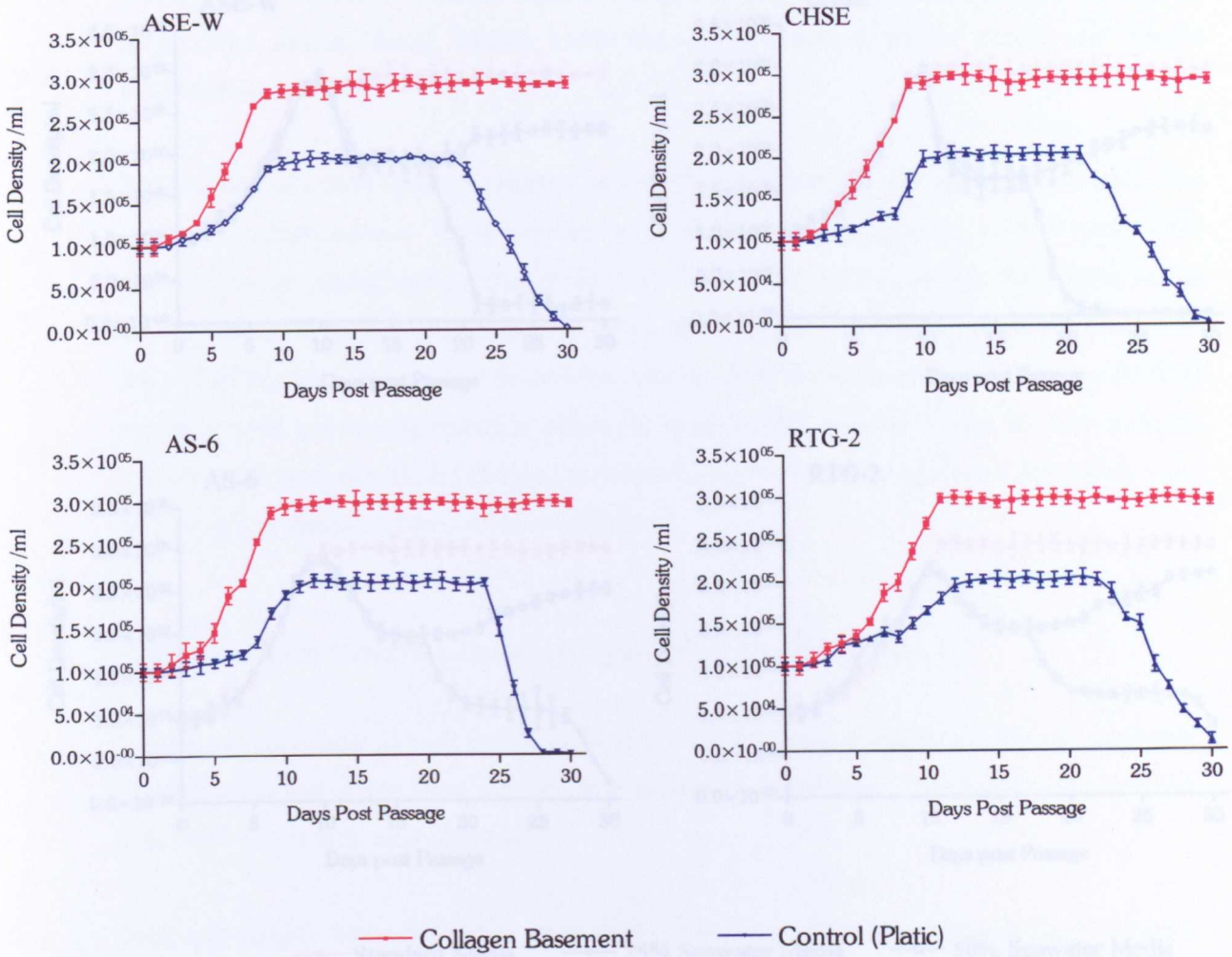


Figure 4.4

The growth of fish cells cultured on plastic culture surface and plastic culture surface coated with type I collagen. Data are mean number of cells per ml (± standard deviation) calculated from duplicate wells per treatment per cell type at 24 hour intervals. All cells were maintained at 22°C in standard culture media (section 2.12).

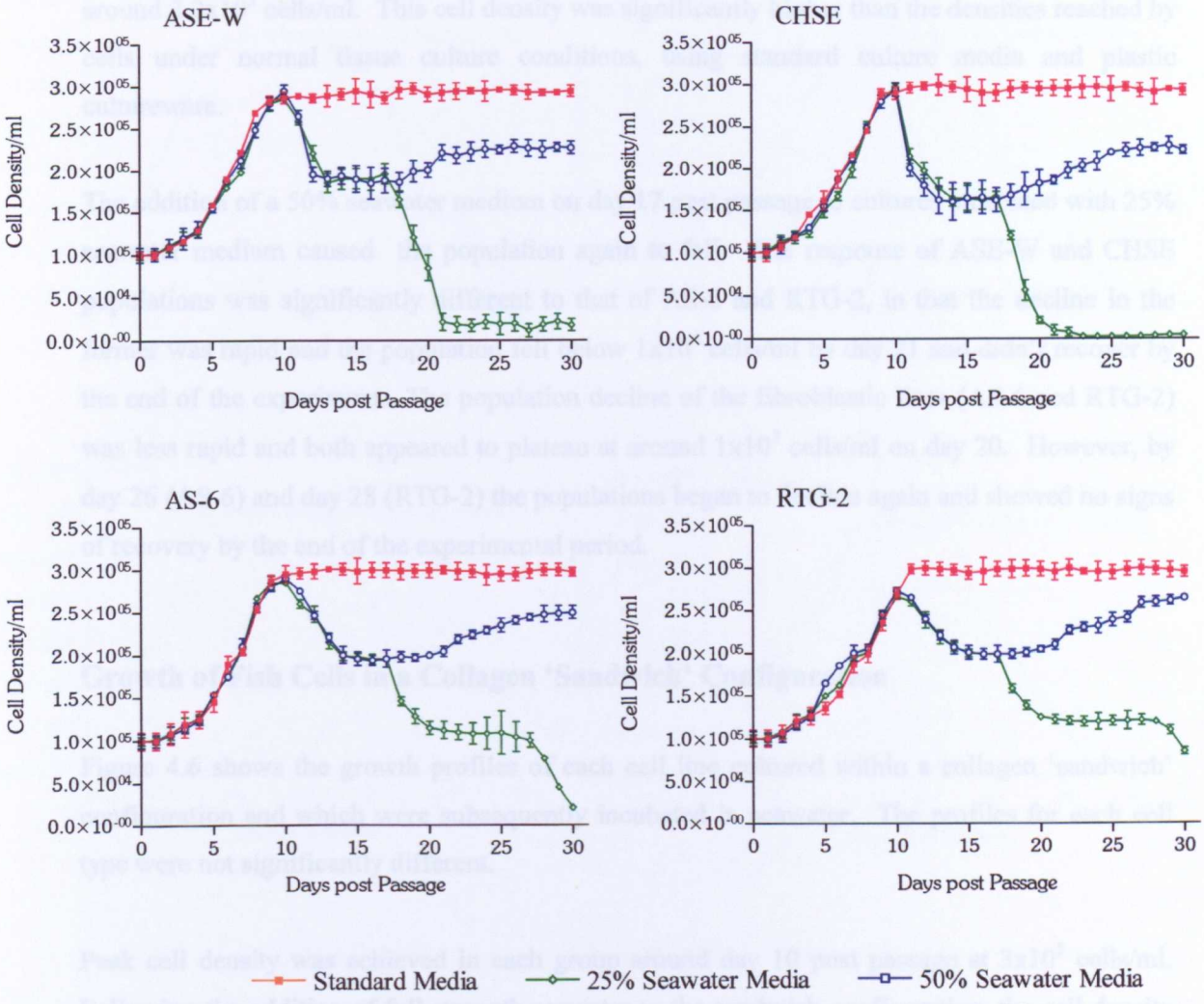


Figure 4.5

The growth of fish cells cultured on plastic culture surfaces coated with type I bovine collagen. Data are mean number of cells (± standard deviation) calculated from duplicate wells per treatment per cell type at 24 hour intervals. All cultures were maintained at 22°C using standard culture media (section 2.12) until day 10 when media in 8 of the 12 culture plates was replaced by a 25% seawater media (500 mOsmol Kg^{-1}). Following a further 7 days of culture the media in 4 of these plates was replaced by a 50% seawater media (800 mOsmol Kg^{-1}). The remaining 4 plates continued to be maintained using the 25% seawater media.

$1.8x10^5$ cells/ml. At this time there was no significant difference in the population level of all cell lines. Following this initial depression in population density, the numbers of viable cells in all cell lines began to rise slowly until beginning to plateau by the end of the experiment at around $2.2x10^5$ cells/ml. This cell density was significantly higher than the densities reached by cells under normal tissue culture conditions, using standard culture media and plastic cultureware.

The addition of a 50% seawater medium on day 17 post passage to cultures incubated with 25% seawater medium caused the population again to fall. The response of ASE-W and CHSE populations was significantly different to that of AS-6 and RTG-2, in that the decline in the former was rapid and the population fell below $1x10^4$ cells/ml by day 21 and didn't recover by the end of the experiment. The population decline of the fibroblastic lines (AS-6 and RTG-2) was less rapid and both appeared to plateau at around $1x10^5$ cells/ml on day 20. However, by day 26 (AS-6) and day 28 (RTG-2) the populations began to decline again and showed no signs of recovery by the end of the experimental period.

Growth of Fish Cells in a Collagen 'Sandwich' Configuration

Figure 4.6 shows the growth profiles of each cell line cultured within a collagen 'sandwich' configuration and which were subsequently incubated in seawater. The profiles for each cell type were not significantly different.

Peak cell density was achieved in each group around day 10 post passage at $3x10^5$ cells/ml. Following the addition of full strength seawater to the sandwich configuration, the cell density was maintained. The cell population of control wells, cultured on collagen substrate but not in the sandwich configuration, showed a sharp decline following the addition of seawater to around half of the peak density by the next day of sampling. The populations continued to fall and cell number fell to below $1x10^4$ cells/ml by day 15 and did not recover.

The viability of cells in the sandwich configuration, however, did not vary from media cultured counterparts after the addition of seawater until around day 18 post passage. Following approximately 6-7 days of stability the cell density of these populations began to decline quite rapidly, although the rates of decline of ASE-W and CHSE lines was significantly less rapid than seen in the AS-6 and RTG-2 lines. At the end of the experimental period all cell populations incubated in seawater were below $1x10^4$ cells/ml.

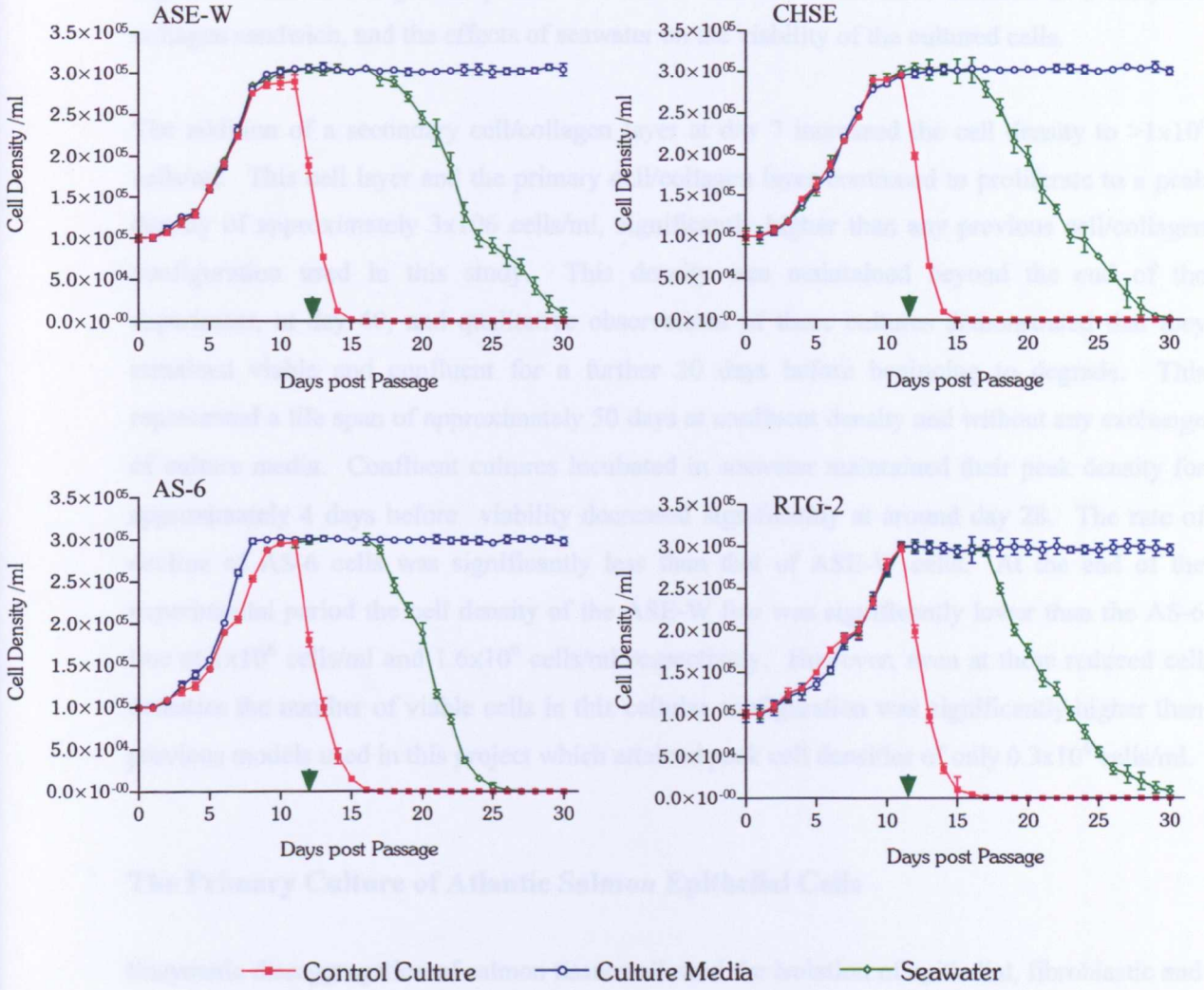


Figure 4.6

The growth of fish cells within a collagen 'sandwich' configuration. Data are mean number of fish cells (± standard deviation) calculated from duplicate wells of each treatment of each cell type at 24 hour intervals. All cultures were maintained at 22°C using standard culture media (section 2.12) until day 12 (marked by the arrow head) when media in half of the culture wells were replaced by seawater (33 ± 2‰). Controls consisted of cells cultured on a collagen substrate but not in the sandwich configuration which were maintained in seawater after day 12.

Growth of Fish Cells Within A Bilayered Collagen 'Sandwich' Configuration

Figure 4.7 shows the growth profile of ASE-W and AS-6 cell lines cultured in a bilayered collagen sandwich, and the effects of seawater on the viability of the cultured cells.

The addition of a secondary cell/collagen layer at day 7 increased the cell density to $>1x10^6$ cells/ml. This cell layer and the primary cell/collagen layer continued to proliferate to a peak density of approximately 3x106 cells/ml, significantly higher than any previous cell/collagen configuration used in this study. This density was maintained beyond the end of the experiment, at day 40, and qualitative observations of these cultures demonstrated that they remained viable and confluent for a further 30 days before beginning to degrade. This represented a life span of approximately 50 days at confluent density and without any exchange of culture media. Confluent cultures incubated in seawater maintained their peak density for approximately 4 days before viability decreased significantly at around day 28. The rate of decline of AS-6 cells was significantly less than that of ASE-W cells. At the end of the experimental period the cell density of the ASE-W line was significantly lower than the AS-6 line at $1x10^6$ cells/ml and $1.6x10^6$ cells/ml respectively. However, even at these reduced cell densities the number of viable cells in this cellular configuration was significantly higher than previous models used in this project which attained peak cell densities of only $0.3x10^6$ cells/ml.

The Primary Culture of Atlantic Salmon Epithelial Cells

Enzymatic dissaggregation of salmon tissues allowed the isolation of epithelial, fibroblastic and dendritic cells. The majority of these isolations were slow growing and short lived, not normally forming confluent monolayers or surviving passage.

However, cells isolated from the opercular membrane, that were epithelial in form, proliferated quite rapidly and in most cases formed confluent monolayers within 3 weeks of primary isolation when maintained at 22°C (figure 4.8). Survival and proliferation following first passage was improved by a reduction in the concentration of foetal calf serum of the culture media. Thereafter, confluency was reached at between 10 and 18 days post passage at approximately $2x10^5$ cells/ml. Cultures persisted normally for between 8 and 12 passages before the ability to reach confluency became diminished. Passage of these sub-confluent cells resulted in low attachment and poor proliferation.

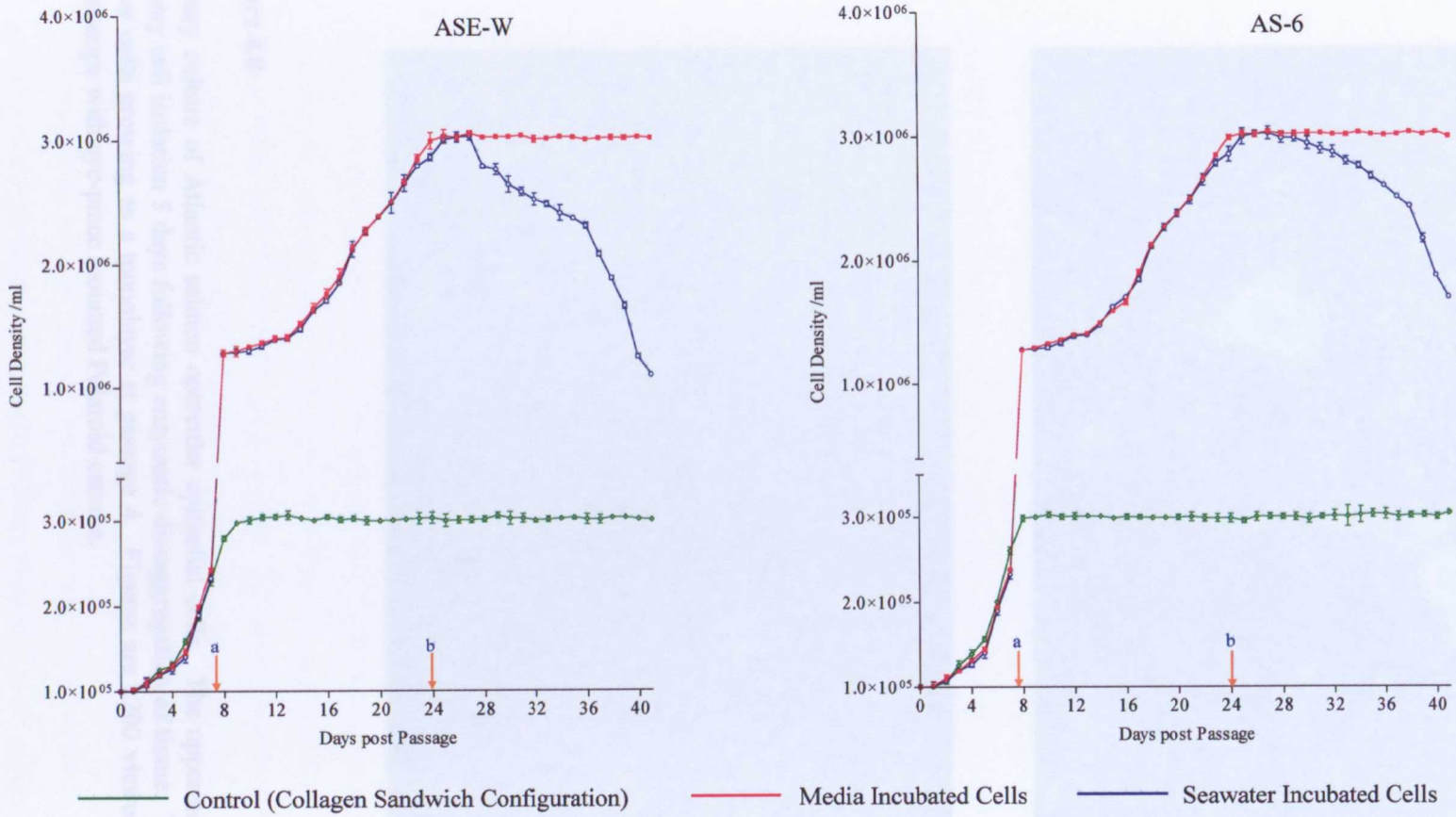


Figure 4.7

The growth of fish cells within a bilayered collagen sandwich configuration. Data are mean number of cells (± standard deviation) calculated from duplicate wells of each treatment of each cell type at 24 hour intervals (n=2). Cells were cultured at 22°C using standard media within a collagen sandwich configuration until day 7 of the experiment (point a) when a cell/collagen layer was added and propagated using standard media. At day 24 (point b) the media from half of the culture wells were replaced by seawater (33 ± 2‰). Culture controls consisted of cells maintained within a collagen sandwich configuration using standard culture media.

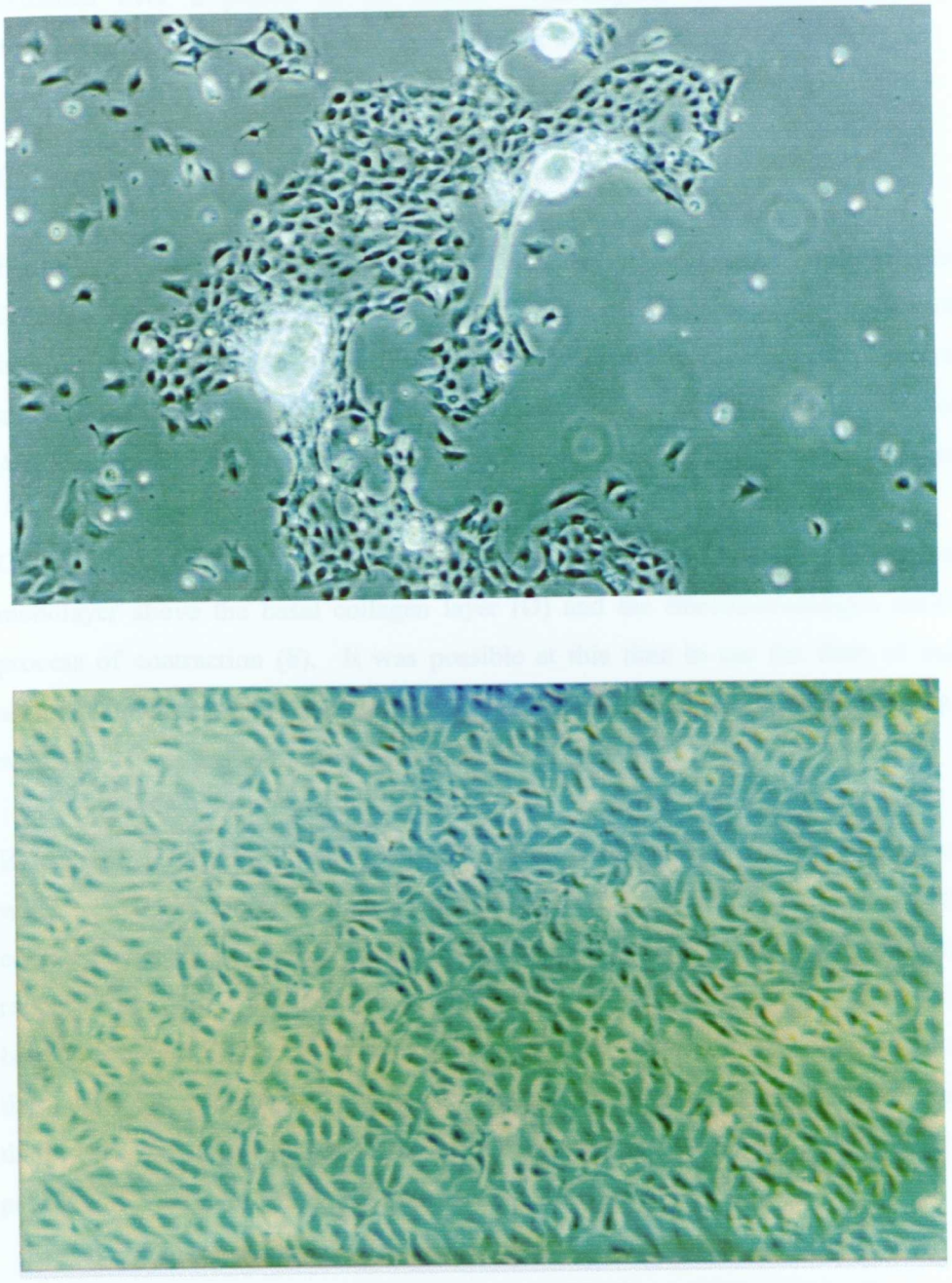


Figure 4.8

Primary culture of Atlantic salmon opercular epithelial cells. The uppermost figure shows primary cell isolation 5 days following enzymatic dissaggregation of tissue. The lower figure shows cells growing as a monolayer at passage 4. Figures are x200 viewed using inverted microscope with eye-piece mounted Polaroid camera.

The maximum number of passages achieved from cells isolated from this tissue was 16 which extended over a period of 40 weeks. Histological evaluation showed no obviously morphological changes that might be associated with the decline in cell viability

Construction of Atlantic Salmon Skin Equivalent

Figure 4.9 shows representative histological views of the process of construction of ASSE. Detailed observations of cell ultrastructure and architecture were not performed and so the identification of differentiated cell types or morphology was not possible. However, the typical strata formation of vertebrate skin could be identified and figure 4.11 allows the comparison of ASSE and normal salmon skin with a diagrammatic representation of ASSE structure.

On day 3 of construction (figure 4.9a) it was possible to visualise the primary fibroblast monolayer above the basal collagen layer (Ø) and the fibroblast/collagen multi-layer in the process of contraction (¥). It was possible at this time to see the form of individual cells arranged unidirectionally, parallel to the basal collagen. Cell and collagen staining were normal with no obvious abnormalities or anomalies.

By day 18 (figure 4.9b) the fibroblasts layer had contracted to form a dermal equivalent (§) and was covered by the epithelial/collagen layer (Ψ) which in this plate was still undergoing contraction. During contraction the individual cells become less distinct as the depth of tissue reduces and cellular proliferation continues. The fibroblast and epithelial layers form distinct layers which are separated by a single layer of darkly staining cells. The origin and nature of these cells is not known. The staining characteristics of the fibroblast layer changed so that there was now little staining of the cytoplasm and only the nuclei were clearly visible. The parallel orientation of these cells appeared less uniform at this time.

Day 35 of construction represents a 'mature' ASSE (figure 4.9c) and was the structure that was used for the *in vitro* culture of *L. salmonis* larvae. At this time the epithelial layer was greatly contracted to approximately 20-30 cells deep. Cell structure was still distinguishable although there was only a low level of nuclear staining. The boundary between epithelial and fibroblast layers was still evident but appeared to consist of darkly staining cells, 3 cells deep (Ω). The cytoplasm of cells in the fibroblast layer were poorly stained, although darkly stained nuclei were apparent. At this time the boundary between the dermal and epidermal layers was not parallel to the basement collagen and its form was reminiscent of papilla-like convolutions seen in vertebrate skin basement membrane and upper dermis.

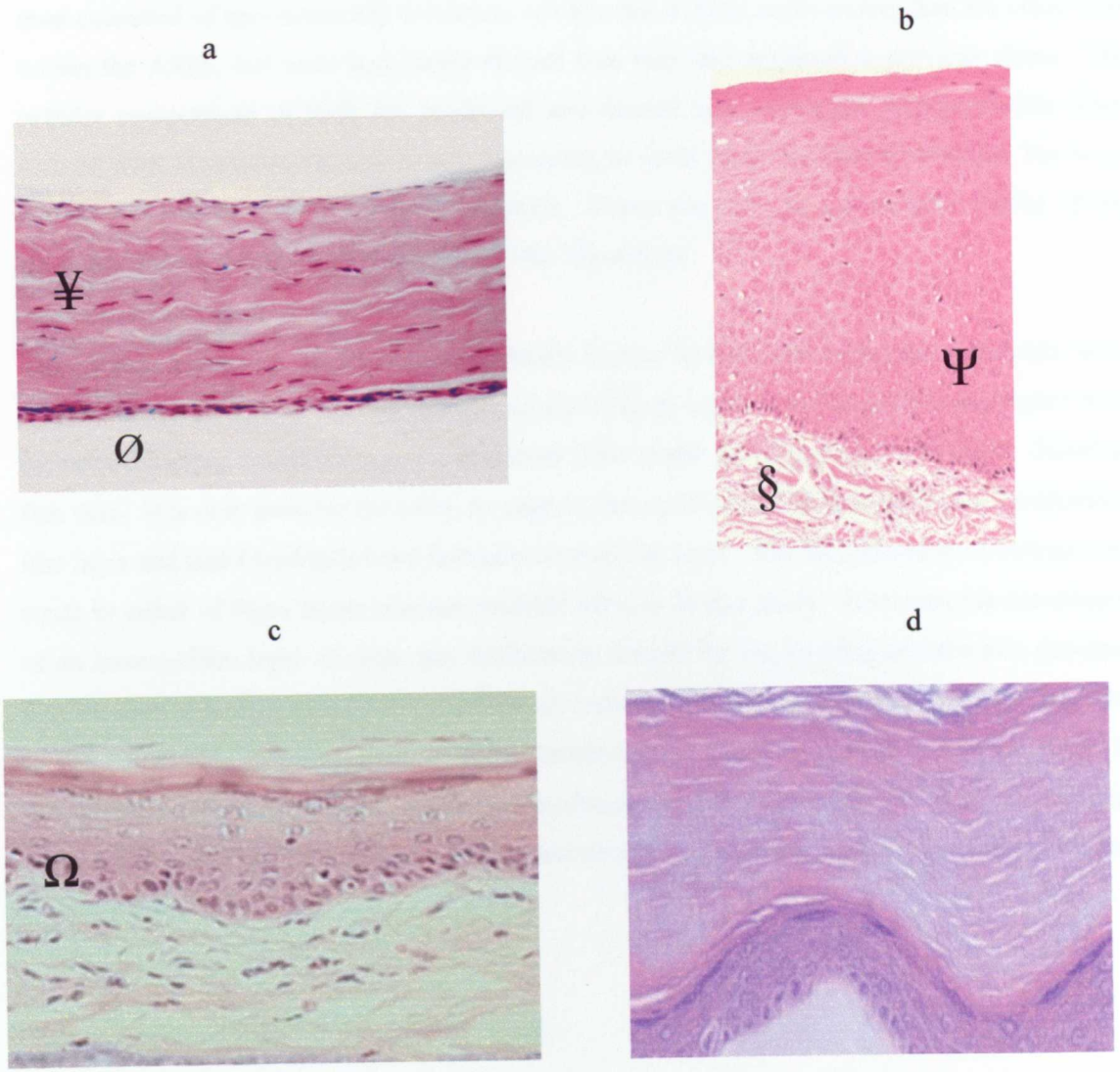


Figure 4.9

Wax sections of the construction of ASSE.

a) day 3: collagen basement, primary fibroblast layer and fibroblast multi-layer (x300, H&E)
b) day 18: fibroblast dermal equivalent and epithelial multi-layer prior to the contraction of the epithelial collagen (x200, H&E)
c) day 35: Mature ASSE: formed dermal equivalent overlaid by epidermal equivalent separated by a region of differentiation cells (x200, H&E)
d) day 48: dermal/epidermal junction characterised by convolutions of approximately 6 layers of darkly staining cells (x400, Giemsa)

At day 48 of construction these papillary convolutions were increasingly obvious and the layer then consisted of approximately 6-8 layers of cells that stained more darkly than the other cells within the ASSE, but were less darkly stained than they had appeared at previous times. The cellular components of both the epidermal and dermal layer were not distinguishable when stained with Haematoxylin and Eosin, appearing as solid bands of stained material, but were more visible when stained with Giemsa stain. There was little or no nuclear staining of the epithelial cells and only limited staining of the fibroblasts.

The layered formation of ASSE is remarkably similar to that seen in normal vertebrate skin. Whilst ultrastructural and biochemical analysis of these layers was not performed, figure 4.11 includes labelling which on a gross structural basis might be compared to the layers found in fish skin. It is only possible however, to suggest that epithelial cells have formed an epidermis-like layer and that fibroblasts have formed a dermal-like layer. The identification of further sub-strata in either of these layers was not possible without further study. However, the occurrence of an intermediate layer of cells, not deliberately formed by the layering construction process, may be seen as a clear indication of cell to cell communication and organisation and its position in the ASSE structure may liken it to the basement membrane of normal vertebrate skin. It should be noted however, that basement membrane *in vivo* is an acellular layer of less than 100nm and which has well defined staining and structural properties which is not the case of the layer within ASSE.

The Growth of Fish Cells Within ASSE

Figure 4.10 shows the growth profile of cells cultured within ASSE.

The initial growth phase of fibroblasts within a collagen multi-layer was not significantly different to previous configurations used in this study. The addition of a secondary layer of epithelial cells however, allowed the increase in peak cell density from approximately $3x10^6$ cells/ml to approximately $5x10^6$ cells/ml following 30 days of growth. These densities were maintained until the end of the experiment on day 60 when incubated in standard culture media. Qualitative observations of ASSE after this time showed the maintenance of structural integrity for a further 30 days before the cell viability declined and the structure began to disintegrate.

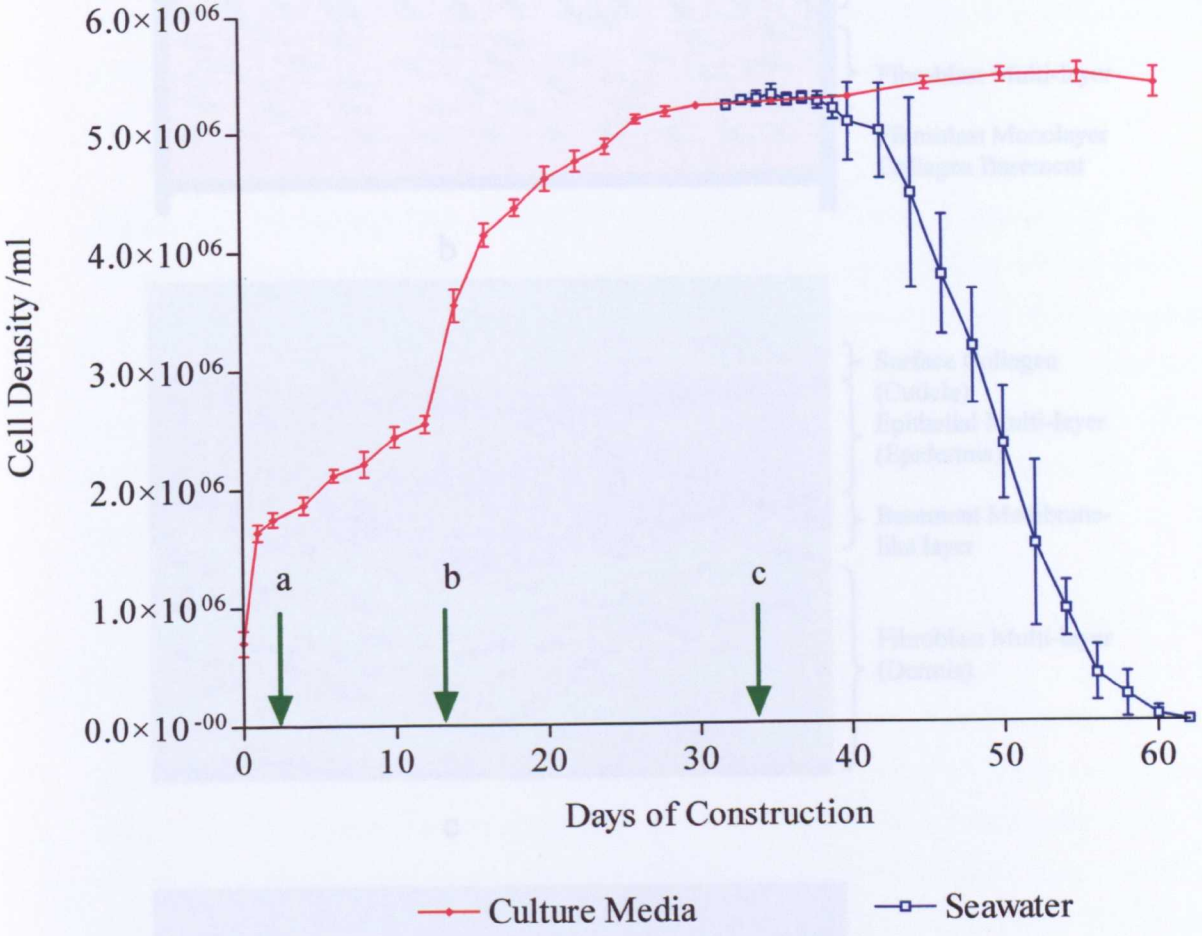


Figure 4.10

The growth of AS-6 fibroblasts and primary cultures of Atlantic salmon epithelial cells within the Atlantic Salmon Skin Equivalent (ASSE) configuration. Data are mean number of cells / ml (± standard deviation) calculated from 8 replicate culture wells at 48 hour intervals. Point **a** marks the addition of the fibroblast/collagen multilayer, and point **b**, the addition of the epithelial/collagen multilayer. Cells were maintained in L-15 culture media until ASSE reached maturity at time point **c** when media was replaced by seawater in half of the culture wells.

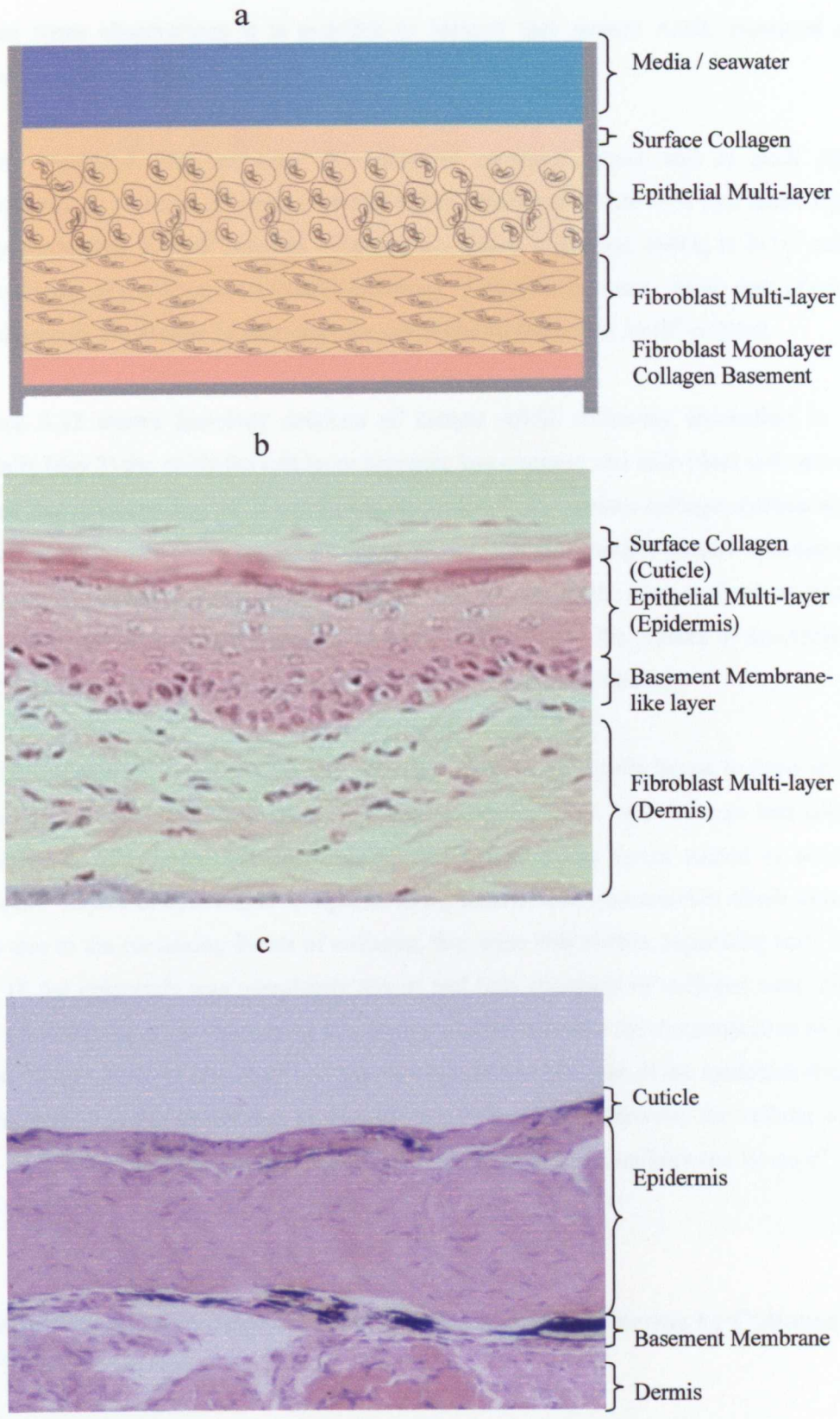


Figure 4.11

Comparative tissue-like structure of ASSE
a) diagrammatic representation of ASSE construction
b) Mature ASSE (day 35 of construction) (x200, H&E)
c) Wax section of skin prepared from the un-scaled head of adult Atlantic salmon (x200, H&E)

Given these observations it is possible to suggest that mature ASSE remained stable for approximately 60 days.

When incubated with seawater the structure remained intact and at peak density for approximately 10 days. However, by day 12 the number of viable cells had fallen significantly to approximately $4.4x10^6$ cells/ml. Viability continued to decline, falling to $3x10^6$ cells/ml (the peak density of previous cell models) following 18 days in seawater. At 60 days of construction (25 days post incubation in seawater) the cell viability fell below $1x10^4$ cells/ml.

Figure 4.12 shows histology sections of mature ASSE following incubation in seawater. Initially (day 3) the epithelial cell layer becomes less compact and individual cells are visible. It seems that seawater ingress is not due to disruption of the surface collagen (which appeared to remain intact at this stage), but more likely due to gaps and fissures present between the ASSE and the sides of the plastic culture wells. This can be seen by the increased rate of death of cells at the sides of the structure compared to that of cells close to the surface of the ASSE. At this time there are no obvious effects on the structures below the epidermis.

Further ingress of seawater occupying the space left by dead cells began to have an increased effect on the epidermal layer (day 10). The structure of this layer became less compact, the number of viable cells decreased rapidly and deeper tissue layers started to become more affected. Layers of cells began to separate along lines of their construction which were apparent only due to the remaining sheets of collagen, that were still visible, separating each layer. By day 18 the epidermis was completely absent and only remnants of collagen were still visible. The fibroblast layer was becoming less compact which allowed the discrimination of individual cells. The progress of cell death and structural disintegration seen in the epidermis was repeated in the dermis to the extent that by day 48 post incubation in seawater the cellular component was all but gone but sheets of collagen that marked the boundaries between layers of cells were still visible.

Indirect Fluorescent Antibody Test of Fibronectin Production by Cultured Fibroblasts.

Figure 4.13 shows the results of IFAT's of fibroblasts cultured in both monolayers and within the ASSE. Twelve slides of each were stained , and the degree of positive staining assessed visually and scored 0 to 4 (0 = no positive staining; 4 = highly positive staining in all fields of view). Control slides containing no cellular material were also assessed.

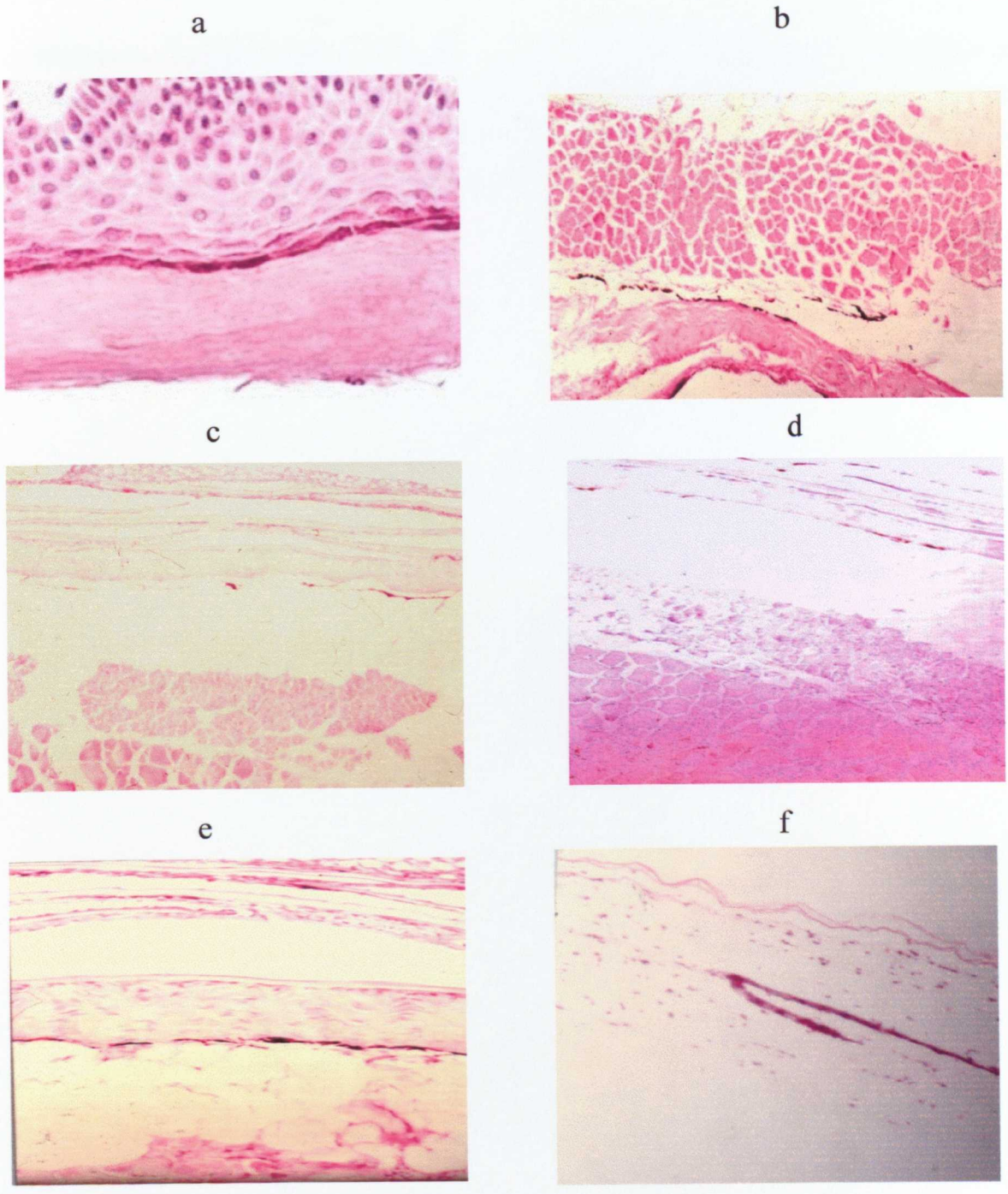


Figure 4.12

Wax sections of ASSE following maintenance in seawater.
a) day 3 (x200, Giemsa)
b) day 10 (x100, H&E)
c) day 18 (x100, H&E)
d) day 28 (x100, Geimsa)
e) day 32 (x100, H&E)
f) day 48 (x100, H&E)

Fibroblasts maintained within ASSE were found to contain significantly higher levels of cytoplasmic and membrane associated fibronectin (mean score 2.4) than monolayer cultured cells (mean score 0.75). Negative controls scored 0.5 which demonstrated that the highly positive response of ASSE fibroblasts is unlikely to be due to false positive staining.

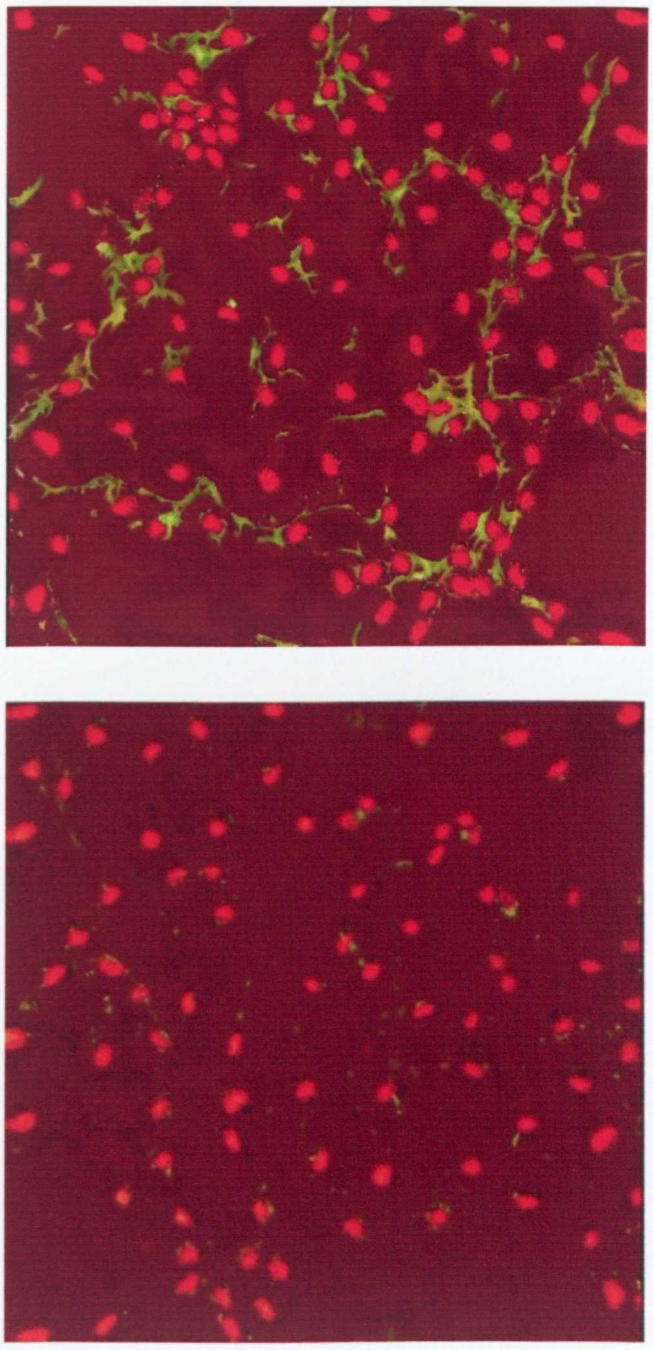


Figure 4.13

Indirect Fluorescent Antibody Test using a mouse anti-human fibronectin monoclonal antibody and FITC conjugated secondary antibody to detect production of fibronectin by cultured salmon fibroblasts. The uppermost figure shows cells isolated following 35 days of culture within the Atlantic Salmon Skin Equivalent (ASSE) and the lower figure displays cells isolated from monolayer culture on a plastic culture surface. Figures are x200 magnification viewed using an Olympus BX60 fluorescent microscope with I3 fluorescent excitation filter (FITC broad band).

DISCUSSION

This chapter investigated the possible strategies that would permit the co-culture of cells from Atlantic salmon with copepodid larvae of *L. salmonis*. It presents data from studies that approached the physiological problem of incompatible maintenance osmolarity in several ways; by adaptation of cultured cells to higher osmolarity; by culturing lice at their lowest tolerance of osmolarity, and by designing substrates of varying complexity to permit maintenance of both cells and parasite at their individual physiological optimum within the same system.

Other authors have succeeded in increasing the culture osmolarity of isolated fish cells above their *in vitro* optimum of approximately 300 mOsm Kg^{-1}. Tocher *et al.* (1994) were able to maintain Atlantic salmon cells at 500 mOsm Kg^{-1} for short periods to permit biochemical investigation of the effects of osmotic stress at the cellular level, whilst Hashimoto *et al.* (1999) were able to gradually adapt cells to 600 mOsm Kg^{-1} for extended periods. In their study, adapted EPC cells were able to proliferate and form confluent monolayers through the selection of tolerant cell populations. In this present study, similar long-term adaptation experiments succeeded in producing populations of salmon cell lines able to survive and grow at 700 mOsm Kg^{-1}, although these had a finite existence of not more than 10 passages. Cells adapted to 500 and 600 mOsm Kg^{-1} had normal morphology but much reduced growth rates. The apparent normality of these cells was not investigated, although physiological changes that permit tolerance to increased osmolarity also seemed to suppress cell apoptosis in EPC cells modified by Hashimoto *et al.* (1999). The extent of the changes that brought about this abnormal functioning are unclear, but if changes to surface topography and biochemical markers, that may have a role in host identification and utilisation by parasites, were altered, then these could affect normal interaction with the host. The unknown consequences of the cultured cells' changed physiology may not make them entirely suitable for use in this project.

However, whilst these limitations were recognised, the successful manipulation of salmon cells to higher osmolarity was discounted as a method for the provision of a suitable culture substrate for *L. salmonis* after examination of copepodid osmotic tolerance. Experiments to investigate the effect of lowered osmolarity to *L. salmonis* larvae showed that at 600 mOsm Kg^{-1} their survival potential was halved. At 1000 mOsm Kg^{-1} (full strength seawater) the 50% population mortality period was 8.27 days, compared to only 4 days at 600 mOsm Kg^{-1}. The obvious physiological difficulties of animals in this environment would not allow the observations of

normal parasite functioning and interaction that was the aim of this study. A model based on a compromise in osmolarity between cultured cells and copepodids was therefore determined to be an unsuitable route to follow.

Functional fish skin possesses both physical and physiological mechanisms which prevent uncontrolled movement of fluids between the animal and its environment (Bullock & Roberts, 1974). These mechanisms allow Atlantic salmon to survive in seawater. Cultured salmon cells however are isolated from other cell types and tissues that influence their organisation into structures that permit osmoregulation and so have a physiological optimum of only around 300 mOsm Kg^{-1}. The use of biological tissue culture substrates such as collagen has in many cases allowed the reorganisation of isolated cells into tissues (Freshney, 1998). This present study adopted these techniques to investigate the potential for the return of osmoregulatory function that might allow the maintenance of established fish cell lines at increased osmolarity.

Collagen is used as a culture substrate for many cell types and has been seen to promote cellular organisation, orientation and return of differentiated functioning (Schor *et al.*, 1979; Nakagawa *et al.*, 1989; Auger *et al.*, 1995) and is easily extracted in large quantities from many animal sources, such as the tails of rats as used in this study (Elsdale & Bard, 1972). Propagation of established salmon cell lines with collagen in several configurations allowed increases in maximum achievable density of cells but did not promote their survival in hyperosmotic or seawater media which both resulted in cell death and greatly reduced cell density. The culture of cells with a collagen coating at the surface, referred to as the sandwich configuration, allowed their extended maintenance in seawater and was a structural rather than a biological solution. Biologically, primary isolations of animal cells produce cell monolayers that retain many differentiated functions in the appropriate environment (Shaw, 1996; Freshney, 1998). It was for this reason that early tissue engineering studies used primary cultures of human cells, although modern techniques now permit the use of established cell lines (Bell *et al.*, 1981a, 1981b; Bell *et al.*, 1991a, 1991b; Parenteau *et al.*, 1991). In the present study primary cultures of Atlantic salmon epithelial cells were co-cultured with an Atlantic salmon established fibroblastic line (AS-6) within a collagen framework to produce Atlantic Salmon Skin Equivalent (ASSE). ASSE was produced following approximately 35 days of culture with standard culture media, after which time it was capable of maintaining its cells density and structural integrity for a further 10 days when cultured in seawater. However, the structure and organisation of cell layers below the surface which were located further from direct contact with seawater, persisted for around 30 days.

There is no precedent for the application of tissue engineered models and materials in exactly this way and so validation of this system against others is not really possible. However, ASSE possesses structural similarities to normal fish skin, in that it has dermal and epidermal layers separated by a basement membrane-like boundary and with a cuticle equivalent layer composed of collagen, in the same way that human living skin equivalents (LSEs) are structurally and biochemically similar to normal human skin. The closest comparative model would therefore appear to be human LSE (Parenteau, 1994).

Structurally, LSE possess the same strata formations as *in vivo* human skin with the same cell types, morphology, function and biochemistry (Eaglstein & Falanga, 1998). These cell types and anatomical structures are produced by the seeding of only two cell types, keratinocytes and fibroblasts, onto extracellular matrix incubated in medium supplemented with numerous growth factors (Bell, *et al.*, 1981b; Parenteau, 1994; Eaglstein & Falanga, 1997). The resulting tissue structure develops wholly from biochemical, developmental and regenerative mechanisms active within the cell/growth factor mixture. The construction of ASSE is very different, primarily in that it is 'constructed' rather than produced by internal processes. Each stratum is laid down as a layer of proliferating cells within, or separated by extracellular matrix, with subsequent layers added at the appropriate time. However, even given the 'artificial' formation methods of the 'tissue' it appears, in some aspects to be at least attempting 'self generation'. This is particularly evident at the fibroblast/epithelial boundary, which changes as the culture progresses. Initially it forms a clearly defined boundary between the bottom and top halves of the construction, running parallel to the bottom of the culture well, but becomes a layer of several cells with darkly staining characteristics surrounded by poorly staining cells projecting into both the dermis and epidermis. Many researchers involved in tissue engineering have identified this region, which *in vivo* represents the basement membrane, as a biochemically active location (Bell *et al.*, 1984; Dubertret *et al.*, 1985; Auger, *et al.*, 1995; Nakazawa *et al.*, 1997; Auger *et al.*, 1999; DuplanPerrat *et al.*, 2000). Cells in this area not only form a physical barrier between upper and lower skin layers but also regulate and facilitate the interaction of keratinocytes and fibroblasts and their products that stimulate changes in cell metabolism, morphology and physiology both above and below the basement membrane (Green *et al.*, 1989; Shaw, 1996). That this region within ASSE appears from histological evidence to be undergoing change suggests a degree of cellular communication and modification. Further investigations may reveal additional evidence of this as well as developing methods that might promote it. The present study made only a preliminary attempt at identifying the return of differentiated functioning within ASSE by measuring the degree of fibronectin production by cultured fibroblasts. Monolayer cultures of fibroblasts produce small amounts of fibronectin but

the dermal layers of ASSE were shown to produce significantly more when assessed by IFAT. It seems that whilst the structure of the current ASSE configuration is constructed rather than generated there are processes working within the structure that indicates at least a limited regenerative capacity.

The tissue-like construction therefore represents an exciting innovation that provides an organotypic culture system that permits maintenance of cells at their optimum within an organised structure that provides a physical barrier against an otherwise cytotoxic aqueous environment. Within the field of tissue engineering this system is unique. Models exist, primarily LSE, that posses a barrier function, but these are to an air interface and not a liquid one (Bell *et al.*, 1981a, 1981b; Lafrance *et al.*, 1995; Eaglstein and Falanga, 1998). LSE were initially developed as wound coverings (Bell, *et al.*, 1981a, 1981b; Eaglstein & Falanga, 1998) but have been successfully used in many medical and research applications (Arnst & Carey, 1998). In the same way it may be possible that ASSE, following further development, could find extensive uses in fish veterinary research as well as stimulating further research into the use of advanced tissue culture technologies in the study of lower vertebrate systems.

THE USE OF ATLANTIC SALMON SKIN EQUIVALENT FOR THE MAINTENANCE OF *L. SALMONIS* LARVAE *IN VITRO*

Chapter 5

INTRODUCTION

THE USE OF ATLANTIC SALMON SKIN EQUIVALENT FOR THE MAINTENANCE OF *L. SALMONIS* LARVAE *IN VITRO*

The maintenance of *L. salmonis* larvae typically involves their incubation in aerated seawater from the point of removal of egg strings from the adult, through the hatch to naupliar stages and metamorphosis to copepodid (Johnson & Albright, 1991). Development beyond this stage does not occur in these systems and maintained in this way copepodids are able to survive for over 7 days following metamorphosis (Tucker *et al.*, 2000), but not normally beyond 12 days (pers. obs.). Those animals maintained without a host substrate for extended periods are known to show a significant decline in energy levels and significantly reduced settlement ability upon finding a host (Tucker, *et al.*, 2000). However, upon successful settlement these animals show rates of survival and development comparable to copepodids of younger age.

The study of *L. salmonis* larvae is therefore not possible past copepodid unless as part of a fish infectivity trial. Whilst valuable information has been derived from this kind of work it is not a biologically controlled environment capable of manipulation and not ideally suited to investigate the specific processes involved in parasite settlement, metamorphosis and interactions with the host which are desired by projects such as this.

Toovey and Lyndon (2000) present a candidate *in vitro* substrate for the culture of *L. salmonis* copepodids which uses primary cultures of cells from Atlantic salmon epidermis grown on an agar substrate. However, they present only preliminary findings and do not include data on the survival of copepodids or the cellular substrate during the incubation of the two. A similar system, using established Atlantic salmon epithelial cells grown on collagen substrates was reported by Balasundaram *et al.* (1995). In this system they report copepodid settlement and survival for between 8-12 days and record the metamorphosis to chalimus I. However, the reproducibility of these results has been questioned following attempts by several groups, including this author. Currently therefore, there are no techniques available that would permit the extended maintenance of copepodids, or allow the development to chalimus larvae *in vitro*.

The maintenance within *in vitro* cellular models of other parasitic organisms is possible and is applied for both research and clinical diagnostic purposes (Cox, 1993). The majority of this

work uses monolayer cultures of cells which are then infected with a particular stage of protozoan parasite such as *Toxoplama* sp. and *Plasmodium* sp., and metazoan helminth parasites (Smyth, 1990). In fish based research such systems are used to study economically important parasites such as *I. multifiliis* and *Gyrodactylus* sp. (Buchmann, 1999; Buchmann *et al.*, 2000). More complex models for the study of more 'demanding' parasites are not available. In this context 'demanding' refers to organisms that have specific developmental requirements and/or require exogenous influences to stimulate normal development, and in this respect *L. salmonis* is a good example. *L. salmonis* is an ectoparasite that requires specific settlement and anchorage conditions within a marine environment. Such requirements means that it is not suitable for maintenance upon monolayer cell cultures within standard tissue culture environments.

Despite the enormous utility of *in vitro* culture models there are a number of challenges that are somewhat unique to such systems. The most significant of these are the requirements of the parasite in terms of the physical, chemical and nutritional characteristics of the environment as well as the need for a specific trigger stimulus, presumably from the host, to allow normal development. In the absence of any of these factors, or the lack of appropriate combination of them the cultured organism may differ in any number of ways from counterparts maintained *in vivo* (Smyth, 1990). However, the practical application of such techniques for the culture of helminth parasites has revealed significant dynamic adaptation of animals to cope with environmental changes *in vitro*. Smyth (1990) reviewed a series of experiments where the human filarial parasite *Brugia malayi* was cultured within a cellular *in vitro* model at a range of serum concentrations whilst causing no significant change to parasite growth, development, morphology or metamorphosis. Conversely, Franke and Weinstein (1984) found that the development of the helminth parasite *Dipetalonena viteae* was highly dependent on both the serum concentration and batch. It is evident then that the culture requirements of even closely related species can differ greatly and that no assumptions should be made of the suitability of a culture system for one organism, based on its success with another. It is important therefore that once an apparently suitable system is designed it is tested to determine the normality of the resulting cultured organisms. In most studies that have examined such animals they have primarily assessed 5-6 criteria of normalness; survival; morphology; growth; metamorphosis; behaviour; subsequent ability to infect the natural host.

Using the criteria of morphology and survival Jura *et al.* (1990) concluded that their preliminary work towards an *in vitro* model for the culture of *E. multiocularis* was successful, as did Weiss *et al.* (1995) who saw no differences in the survival, morphology and metamorphosis of *T.*

gondii bradyzoites maintained within a cellular system. On the other hand Islam *et al.* (1999) found that *Stronglyoides venezuelensis* from *in vitro* culture had significantly greater body length than those obtained from faecal samples, and Bonnet *et al.* (2000) reported reduced rates of feeding by mosquitoes maintained on an artificial substrate with the consequent reduction in the transmission and survival of *Plasmodium falciparum* carried by the insects. *In vitro* systems do therefore have their drawbacks and limitations, but so long as those working with them are aware of these, they can provide valuable diagnostic and research tools, in the same way as monolayer cell culture with its significant limitations has become a standard *in vitro* tool over the last half century.

Various methods of supplementation of the culture systems have been employed in attempts to initiate normal development or functioning of the cultured organisms (Smyth, 1990). Supplementation may take the form of specific chemicals known to stimulate the desired response from the parasite, such as fatty acids used by Jura *et al.* (1990) or hormones, or their precursors reviewed by Freshney (1998) and Smyth (1990). Alternatively more broad range biochemical substances such as serum, mucus and homogenised tissues can be used (Freshney, 1998). These substances contain large concentrations of primarily proteins but also other metabolites and biochemically active products which can provide either the required nutritional components or specific affector molecules that are absent from the culture system and which may provide the stimulus to permit successful culture. This has been demonstrated most recently by Nielsen *et al.* (2000) who report improved development of *I. multifiliis in vitro* following the addition of rainbow trout mucus and serum. They did not though investigate further whether their addition to the culture environment had other less obvious effects on the parasite.

In this project the *in vitro* system on trial is supplemented with mucus and peptone from Atlantic salmon but also with DL-methionine. Methionine is known to both catalyse the synthesis of methyl farnesoate which in crustaceans is comparable to the arthropod juvenile growth hormone III (JH), and to be a necessary component of juvenile hormone binding proteins (JHBP) in insects (Wainwright *et al.*, 1996). The hormone in the crab *Cancer pagarus* has been shown to be associated with ovarian maturation in adults and with moulting and metamorphosis in juveniles, as well as with the stimulation of ecdysteroid moulting hormone by tissue *in vitro*, a process which is limited by the exclusion of exogenous methionine (Yudin *et al.*, 1980). JHBPs belong to the protein superfamily of arthropods known as haxamerins which have numerous sub-families with wide ranging roles. One JHBP class contains 10 mol % methionine and is associated with the transports of JH to the target tissues in insects (Telfer &

Kunkel, 1991). The methionine component of the ASSE cellular substrate used in this study is not known and so an exogenous source of the chemical was provided should it be required to permit normal larval development and metamorphosis.

MATERIALS AND METHODS

Chapter 5 will assess both the suitability of the Atlantic Salmon Skin Equivalent described in chapter 4 to the culture of *L. salmonis* copepodid larvae, and its applicability as a fish skin substitute *in vitro* tool for other, non-culture experiments.

The culture process is described in section 2.27. Subsequent measurements will firstly determine the effects of louse culture upon the ASSE substrate (section 2.27.3), before examining the performance of copepodids within the culture system (section 2.28). Performance criteria include the measurement of louse survival (2.28.1), observations of behaviour (section 2.28.2) which also includes the measurement of copepodid feeding on the cellular substrate (section 2.28.3), and measurement of louse development and growth (section 2.28.4). These same criteria will also be used to determine the effects on louse performance of ASSE supplementation with salmon mucus, salmon peptone and methionine (section 2.29).

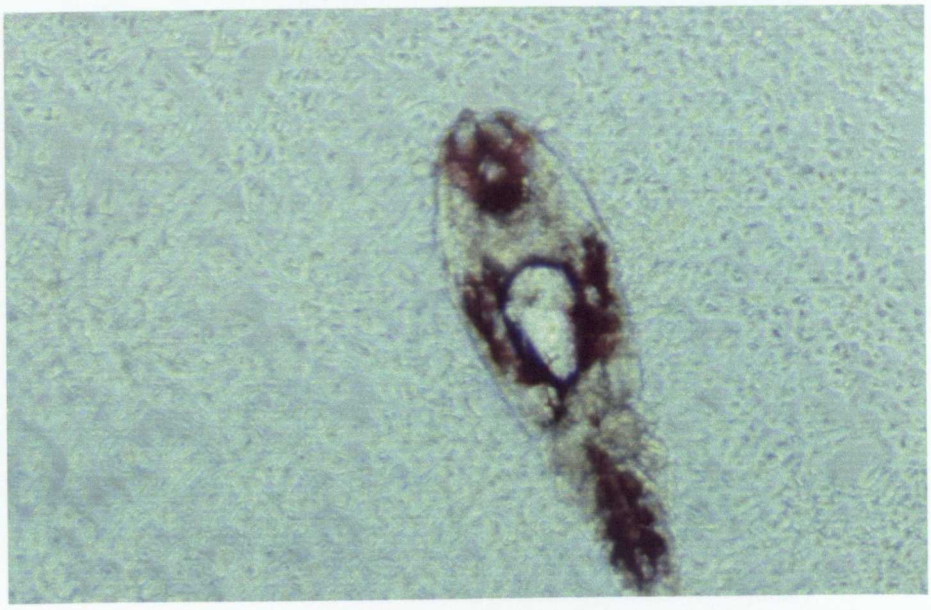
In a related experiment the use of ASSE in short term *L. salmonis* larval assays, rather than in extended culture experiments, will be investigated. In this study its effectiveness in measuring the host selection behaviour by copepodids will be tested in a series of modified 'choice chamber' experiments (section 2.30).

RESULTS

The Effects of Copepodid Culture on ASSE

Figure 5.1 shows typical copepodid orientation on the surface of ASSE during burrowing and settled behaviour. During the initial settlement phase following incubation burrowing behaviour caused extensive damage to the surface collagen and the epithelial layer in the areas immediately surrounding the louse. This was particularly evident in the region of the second antennae (figure 5.3) and posterior swimming legs which repeatedly grapple with the surface until sufficient anchorage was achieved. The majority of individuals observed spent significant periods walking across the substrate surface in the burrowing posture apparently attempting to penetrate the collagen layer with the carapace. Frequently lines of 'scoring' were seen on the surface of the ASSE that tracked the movements of copepodids from their initial contact point until their eventual settlement situation. Burrowing behaviour continued in this location and was responsible for the majority of damage to the surface layers. Removal of copepodids from these areas showed little or no damage to the substrate immediately below the body and none that corresponded to the position of the mouth parts suggesting no attempts at feeding on the surface at this time.

When settled, the periods of burrowing were infrequent and little additional damage to the area around the louse was thought to be caused. However, damage to the substrate integrity from previous burrowing behaviour permitted the ingress of seawater, and cell death continued as a result of osmotic changes. The extent of osmotic damage could be seen microscopically as clear patches of acellular collagen fibres and these areas increased as the culture cycle progressed. At this time the layers beneath the epithelial multi-layer appeared intact and unaffected. Following the cessation of burrowing the louse adopted the typical settled position seen in figure 5.1a and was anchored to the surface both by the penetration of the frontal plate of the cephalothorax and the second antennae through the surface layers (figure 5.3). The small area of cells under the louse, previously seen to be unaffected by the louse activity, at this time, began to be cleared. The mechanism of clearing was likely in part to be osmotic damage caused by seawater ingress, but the location of the first cleared cells was towards the anterior of the louse which corresponds to the position of the mouth parts which may suggest damage caused by copepodid feeding.



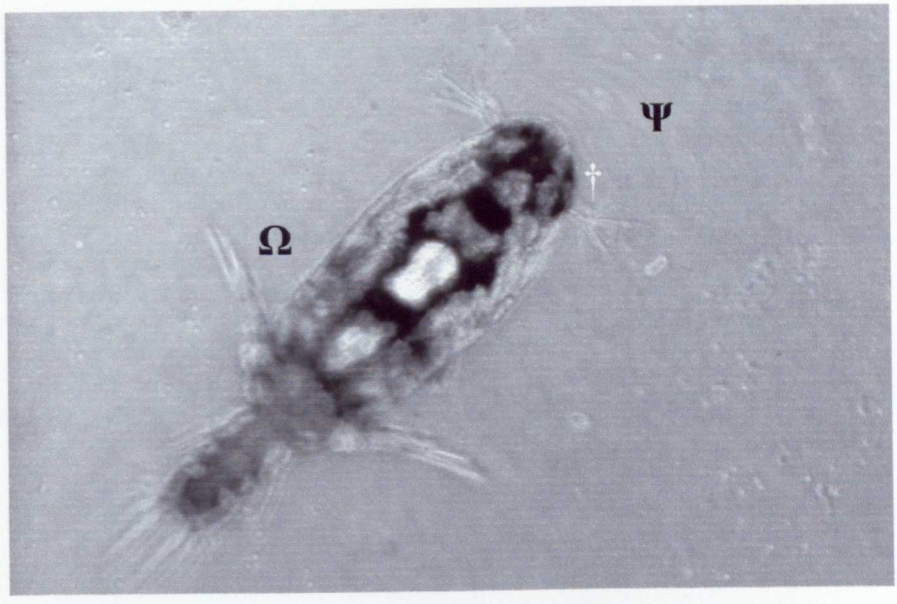


Figure 5.1

Typical orientation of copepodids maintained with ASSE. The upper figure shows a copepodids settled on the culture surface 4 days post incubation. The lower figure shows a copepodid, 2 days post incubation, adopting the typical burrowing posture with leading edge of carapace embedded into surface of the ASSE (†), the abdominal segments elevated, and the swimming legs positioned beyond the marginal of the cephalothorax providing a forward thrusting motion (Ω). 'Waves' of distortion of the culture surface, indicative of this forward motion, can be clearly seen anterior to the cephalic region (Ψ).

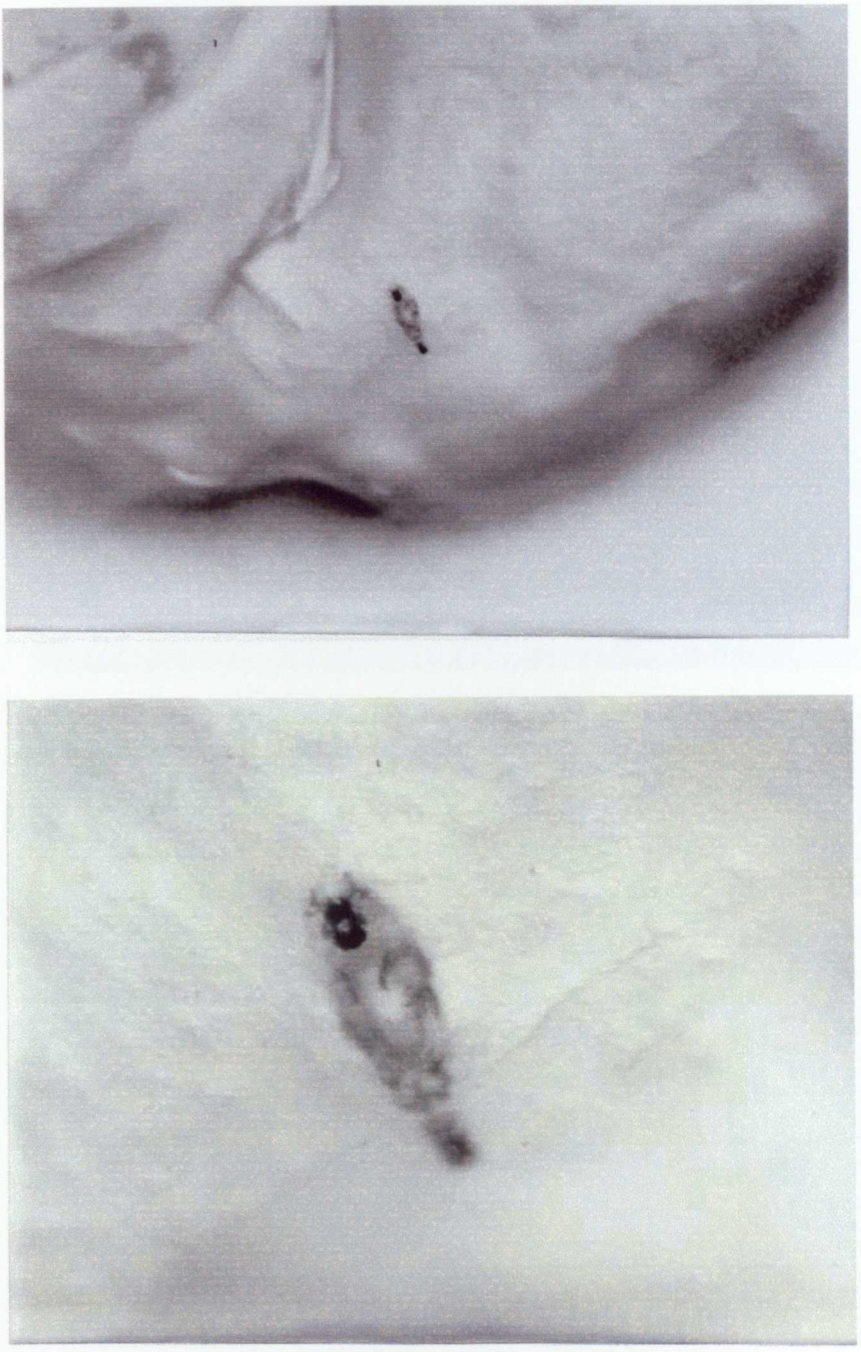


Figure 5.2

Low power view of copepodids settled at the surface of the ASSE. These specimens were fixed in situ by the addition of 10% buffered formal saline and the ASSE removed to be viewed. The ridged surface of the skin equivalent can be clearly seen, as can the typical settled pose of copepodids after incubation with ASSE for longer than 3 days.

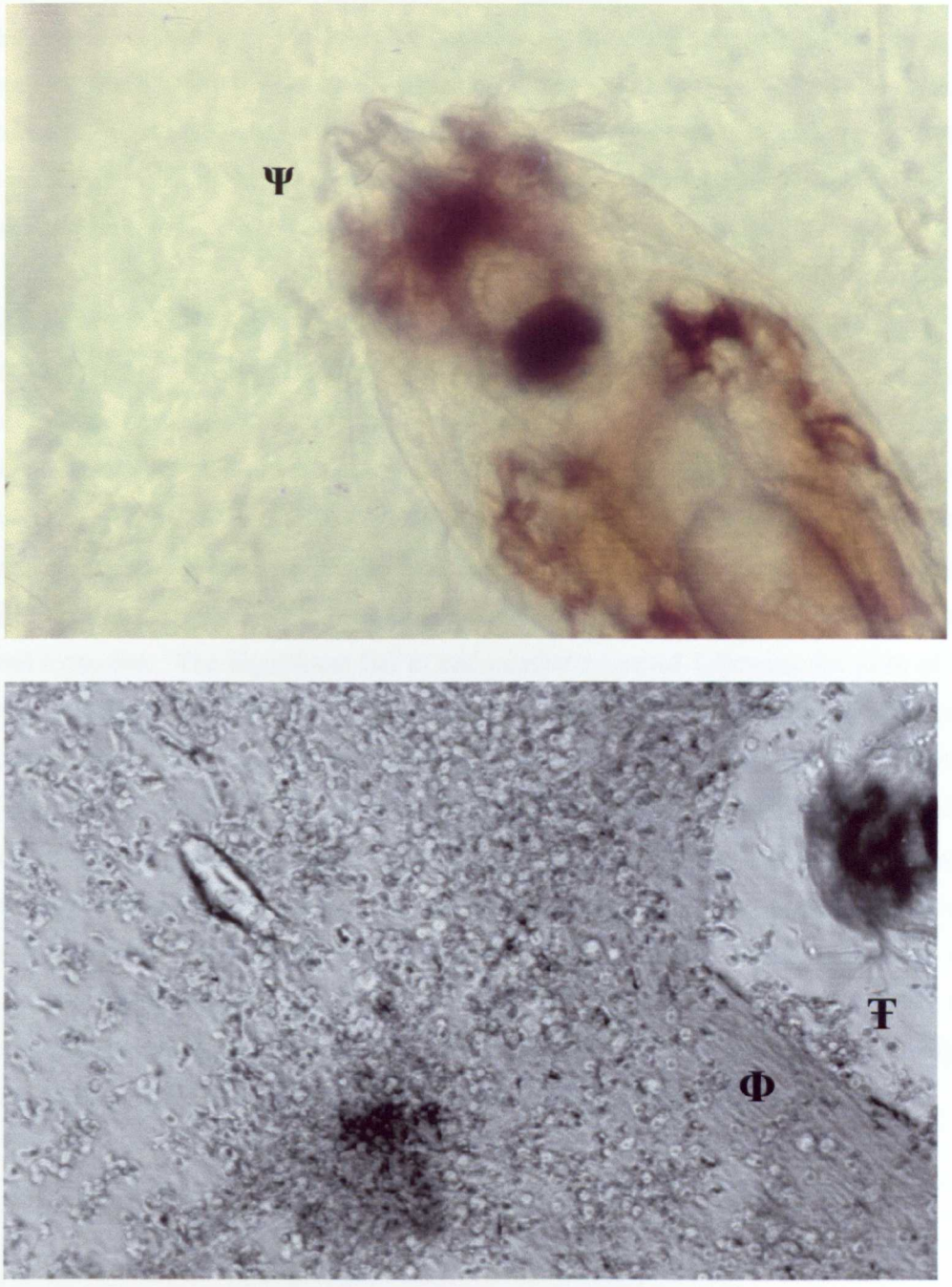


Figure 5.3

The upper figure shows a dorsal view of the attachment of the copepodid to the surface of the ASSE. The second antennae (Ψ) are positioned anterior to the cephalic region and are embedded through the surface of the skin equivalent. The lower figure shows the cephalic region (far right) of a copepodid incubated with ASSE for 4 days and the zone of clearing of surrounding cells (Ŧ). Tissue further from this zone is also affected and shrinkage of collagen/cell layers can be seen immediately following the clearance zone (Φ), followed by cells not yet affected by either the physical damage caused by louse burrowing activity or by ingress of seawater through the damaged surface layers.

As the cycle continued the area beneath the settled louse became completely cleared of cells as did a large area surrounding. The louse typically did not detach and resettle following the clearance of these areas and made no apparent attempts to burrow down to the underlying cellular areas which remained relatively intact. The body was kept in position by collagen fibres not affected by either the mechanical or physiological damage to the ASSE and rarely were animals seen embedded below the surface of the surrounding tissue.

As the area of damage increased, the impact on the underlying cells caused cell death and a rapid reduction in cell viability and liberation of aggregations of cells into the media. Figure 5.4 presents cell survival data that describes empirically the observations made above. The large variation between replicate wells does not permit statistical discrimination of differences between data, and not until 5 days post incubation with lice in seawater did the fall in cell viability become significant. Following louse incubation there was an initial period where the behaviour had little impact on the total cell number, even though damage to the ASSE surface appeared extensive. The significant fall in cell number occurred following the peak of louse burrowing behaviour and resulted from the ingress of seawater into the structure. The profile was significantly different to that of ASSE incubated with seawater alone and it is evident that louse activity had increased the speed of the process. After day 3 there was an almost exponential fall in cell viability that dropped below $2x10^6$ cells/ml (the density of the bilayered collagen sandwich previously trialled in this study). After 13-14 days post incubation the culture environment consisted of floating aggregations of cells and collagen, although some areas did still remain surprisingly intact and maintained at least their layered structure when viewed following histological processing. It is primarily to these areas that surviving lice remained attached, although individuals were observed on floating cell aggregations.

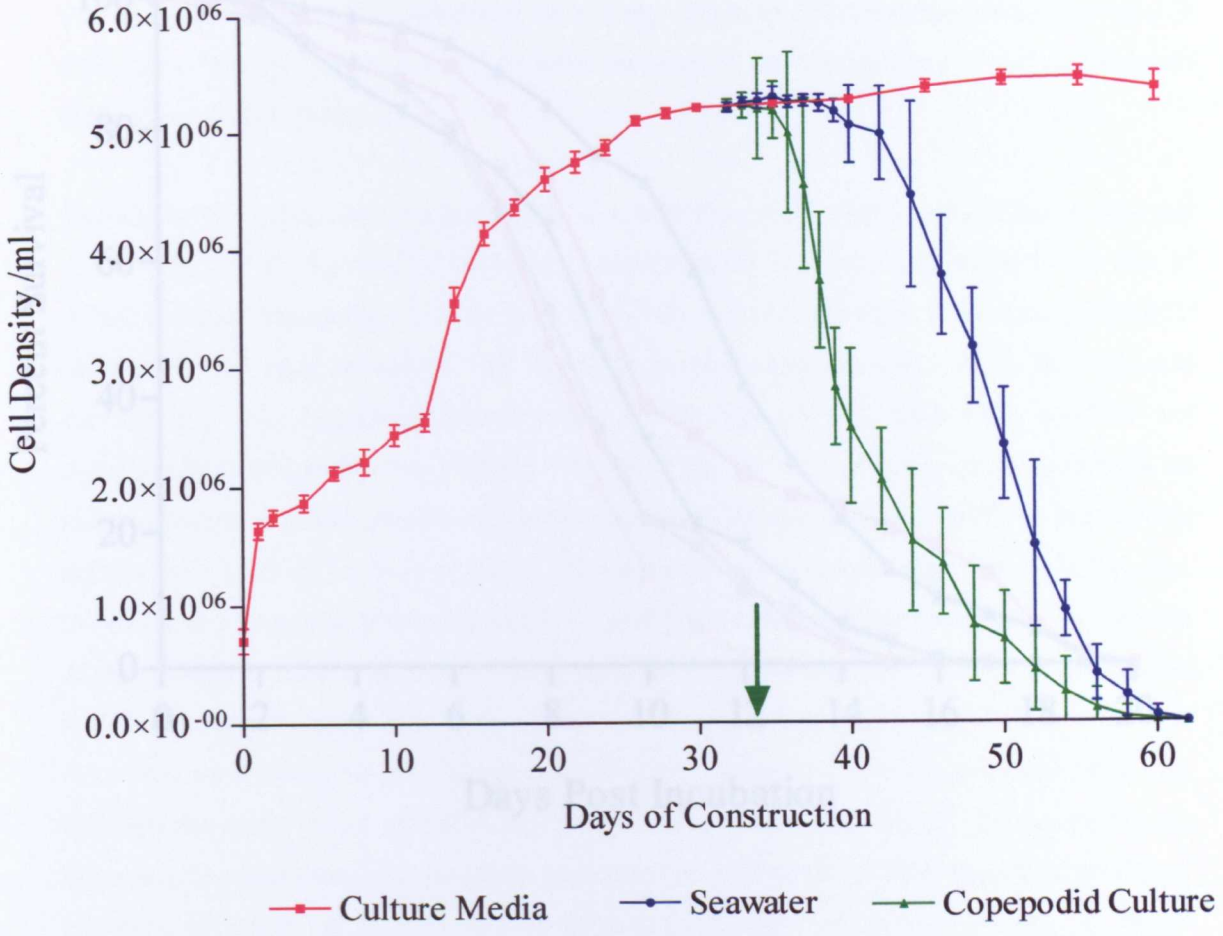


Figure 5.4

The effects of copepodid incubation on the viability of ASSE. Data are mean number of cells per ml (± standard deviation) calculated from duplicate wells per treatment at 24 hour intervals. The arrow indicates the point at which culture media was replaced by seawater in 2 of the treatment groups, and also the point at which copepodids were added to wells of the group labelled 'Copepodid culture'. Prior to this point, all groups were incubated in standard L-15 culture media.

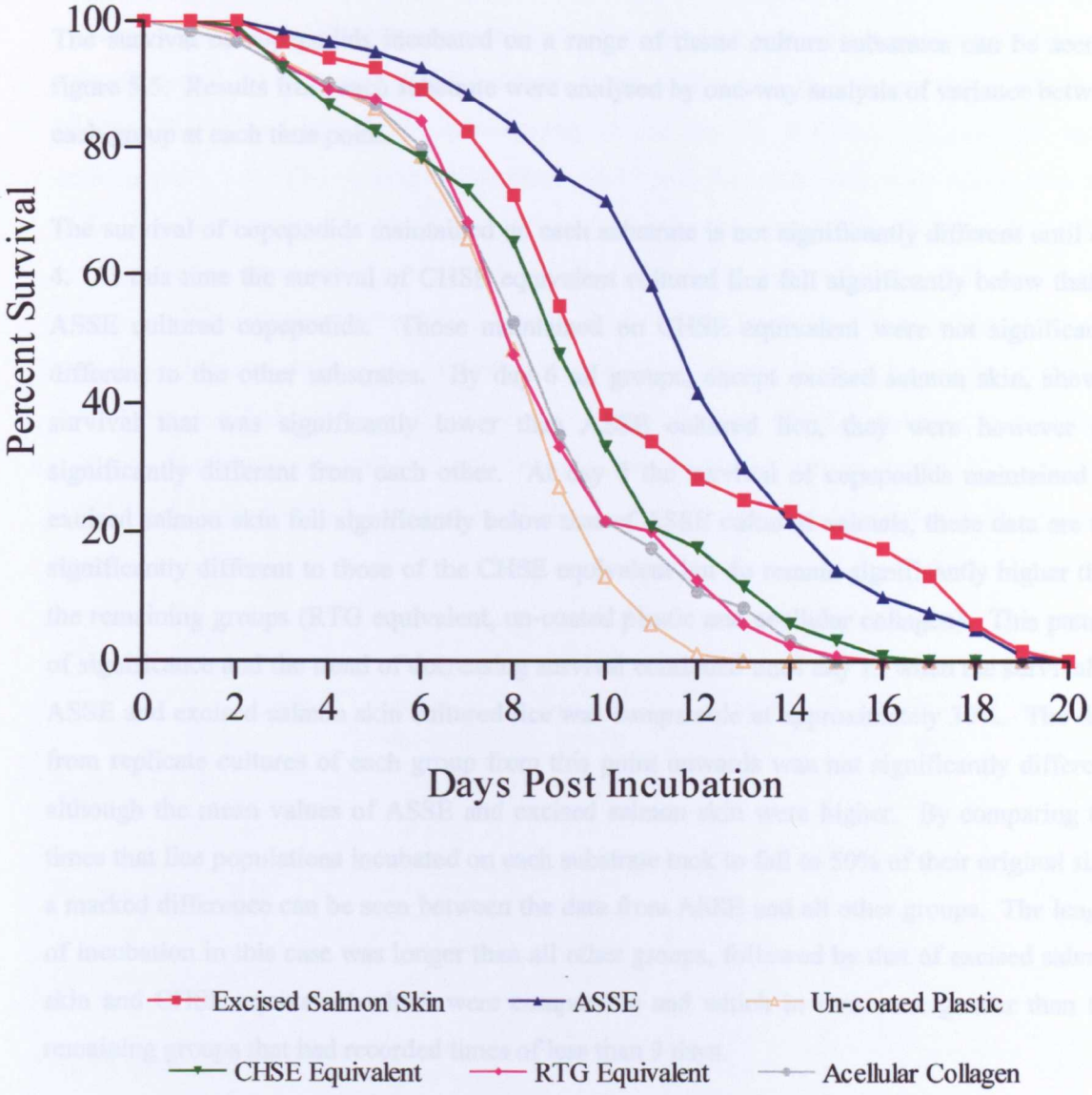


Figure 5.5

Survival of L. salmonis copepodids on tissue culture substrates. Data are mean percent survival, excluding error bars to allow easier comparison of data at each time point for each substrate. n= 96, N=960, (n= number of observations per substrate, and N= number of copepodids observed per substrate). A complete data set for each substrate, including error bars can be seen in the appendices to this thesis.

Measurement of Copepodid Survival

The survival of copepodids incubated on a range of tissue culture substrates can be seen in figure 5.5. Results from each substrate were analysed by one-way analysis of variance between each group at each time point.

The survival of copepodids maintained on each substrate is not significantly different until day 4. At this time the survival of CHSE equivalent cultured lice fell significantly below that of ASSE cultured copepodids. Those maintained on CHSE equivalent were not significantly different to the other substrates. By day 6 all groups, except excised salmon skin, showed survival that was significantly lower than ASSE cultured lice, they were however not significantly different from each other. At day 8 the survival of copepodids maintained on excised salmon skin fell significantly below that of ASSE cultured animals, these data are not significantly different to those of the CHSE equivalent but do remain significantly higher than the remaining groups (RTG equivalent, un-coated plastic and acellular collagen). This pattern of significance and the trend of decreasing survival continued until day 13 when the survival of ASSE and excised salmon skin cultured lice was comparable at approximately 30%. The data from replicate cultures of each group from this point onwards was not significantly different, although the mean values of ASSE and excised salmon skin were higher. By comparing the times that lice populations incubated on each substrate took to fall to 50% of their original size, a marked difference can be seen between the data from ASSE and all other groups. The length of incubation in this case was longer than all other groups, followed by that of excised salmon skin and CHSE equivalent which were comparable and which in turn were greater than the remaining groups that had recorded times of less than 9 days.

Substrate	50% Survival Period (Days) †	Maximum Recorded Survival (Days) ‡
ASSE	12.0	19
Excised Salmon Skin	9.6	19
Un-coated Plastic	8.2	13
CHSE Equivalent	9.1	16
RTG Equivalent	8.5	15
Acellular Collagen	8.4	14

Table 5.1 Duration of survival of louse populations incubated on culture substrates.

† Data are taken from mean survival of copepodids incubated on each substrate during 4 replicate culture cycles. ‡ Data represent the maximum survival time of a single copepodid in any one of the culture cycles.

Pearsons rank correlation was used to describe the relationship between data from each group within 3 time periods of this experiment that were marked by changes in the rate of copepodid mortality (and can be easily identified from charts in figure 5.5); between day 0 and day 9; between day 10 and day 15; and between day 16 and day 20. Results of this analysis can be seen in table 5.2. The correlation between ASSE and the other substrates within each time period was poor with only data from excised salmon skin correlating closely in the first period. No relationship between these data sets exists in the middle period, but the data improves to give a co-efficient of approximately 0.7 in the final period. Analysis of the correlation between the other substrates shows a good relationship between all during the first 2 periods. In the final period, the survival of lice maintained on RTG equivalent, CHSE equivalent and acellular collagen show high correlation, as do those of lice incubated with excised salmon skin and ASSE with only data from un-coated plastic groups showing no correlation with any other group.

	ASSE	Excised salmon skin	Un-coated plastic	CHSE equivalent	RTG equivalent	Acellular collagen
ASSE	----------	0.948	0.561	0.492	0.687	0.572
	----------	0.320	0.080	0.298	0.121	0.130
	----------	0.692	0.000	0.108	0.049	0.074
Excised salmon skin		----------	0.655	0.552	0.787	0.760
		----------	0.335	0.891	0.545	0.582
		----------	0.000	0.022	0.001	0.008
Un-coated plastic			----------	0.874	0.971	0.982
			----------	0.525	0.870	0.925
			----------	0.091	0.212	0.268
CHSE equivalent				----------	0.932	0.844
				----------	0.622	0.511
				----------	0.877	0.924
RTG Equivalent					----------	0.976
					----------	0.955
					----------	0.969
Acellular collagen						----------

Table 5.2 Results of Pearsons Rank Correlation analysis of copepodid survival on culture substrates. Data are grouped, corresponding to 3 periods during the culture cycle; day 0 to day 9 (Black); day 10 to day 15 (Red); day 16 to day 20 (Green).

Using the 50% survival period allows a simplified summary of the relative performance of animals maintained in each system. The length of incubation in ASSE was significantly longer than any other group, followed by that of excised salmon skin which in turn was much greater than the time in remaining groups which each show comparable times of <9 days.

At no time during copepodid culture did metamorphosis to chalimus I occur, and no individual was observed to be in the process of metamorphosis or frontal filament production.

Measurement of Copepodid Behaviour In Culture

Figure 5.6 shows the pattern of behaviour of copepodids incubated on tissue culture substrates. In all populations maintained on cellular substrates the trend was of increasing periods of settled behaviour, and decreasing time spent performing burrowing and swimming behaviour. The pattern of behaviour on un-coated plastic culture surfaces was different in that animals spent the majority of time in the water column which continued throughout the experimental period with sporadic and infrequent performance of the other behaviours. Copepodids on acellular collagen substrates did show a general trend of decreased swimming behaviour and increases in

the time spent settled on the culture surface, however from 30 hours post incubation until the end of the experiment the times spent performing each behaviour were approximately equal.

One-way analysis of variance and paired two sample t-tests performed on the data from copepodids on cellular substrates shows no significant differences at any time point between behaviour on excised salmon skin and ASSE. In these cases the majority of the time was spent performing settled behaviour, from the first observation point which increases in proportion until the end of the experiment. Swimming behaviour was of short duration and intermittent and comprised less than 5% of the observation period by the second sampling point (20 hours). Similarly burrowing behaviour occupied less than 30% of the first observation point and decreased to less than 15% by 30 hours post incubation. This behaviour was also seen in copepodids that were apparently settled but were readjusting their position on the culture substrate and was recorded as forming less than 5% of behaviour by 44 hours post incubation.

The behaviour of copepodids on CHSE equivalent, whilst following the general trends of the cellular substrates described previously was significantly different to that seen on both excised salmon skin and ASSE. It was however, not significantly different to that recorded from copepodids maintained on RTG equivalent. In both these cases the proportion of time spent performing each behaviour was significantly different throughout the experiment until the final observation point (60 hours post incubation). Whilst settled behaviour predominated in excised salmon skin and ASSE from the first time point, the first 2 observations in CHSE and RTG equivalents showed that swimming behaviour was dominant with a change to settled behaviour

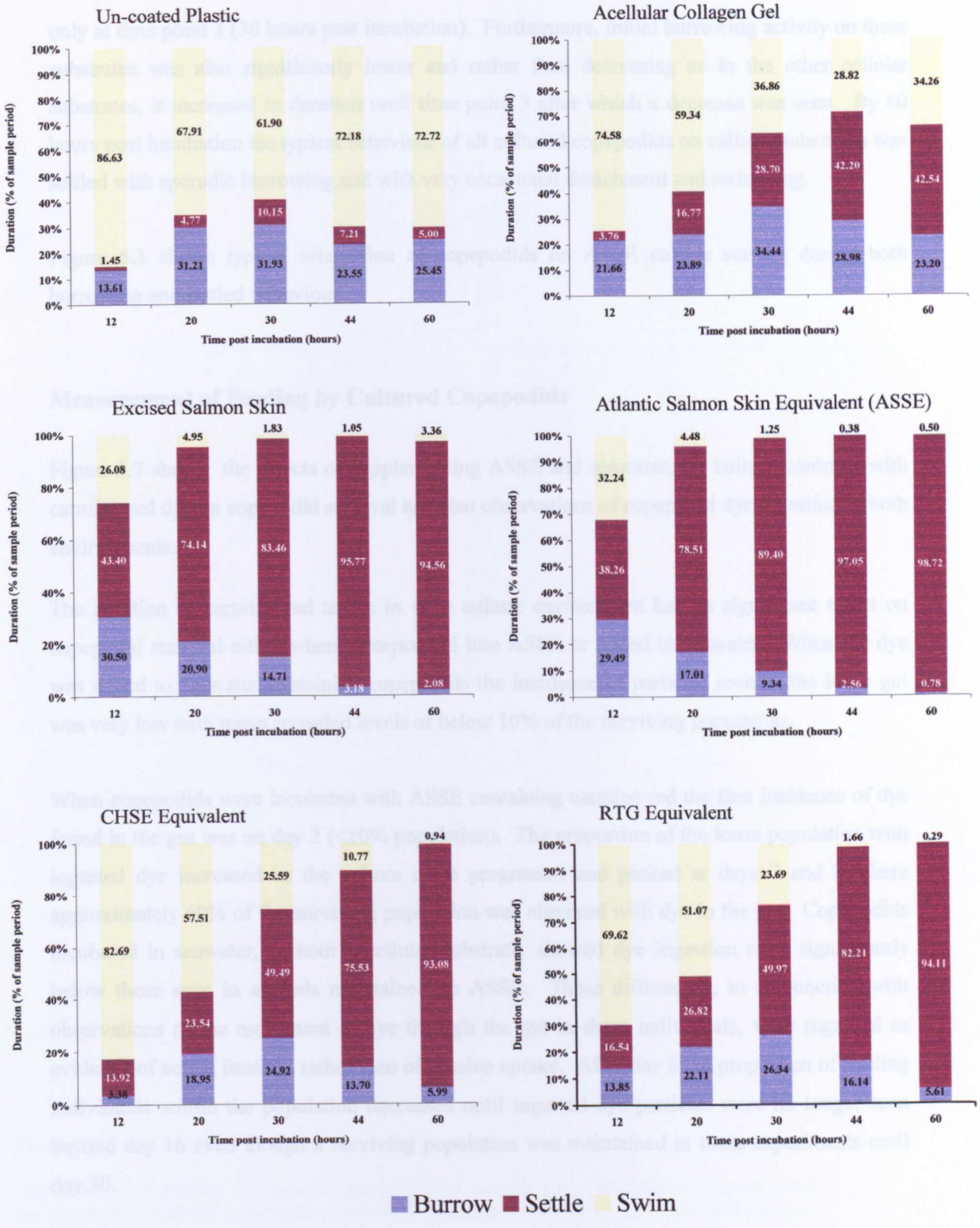


Figure 5.6

The proportion of time spent by copepodids cultured on each tissue culture substrate performing, burrowing, settling and swimming behaviours. Data are mean % of time, n=4 at each time point for each substrate.

only at time point 3 (30 hours post incubation). Furthermore, initial burrowing activity on these substrates was also significantly lower and rather than decreasing as in the other cellular substrates, it increased in duration until time point 3 after which a decrease was seen. By 60 hours post incubation the typical behaviour of all cultured copepodids on cellular substrates was settled with sporadic burrowing and with very occasional detachment and swimming.

Figure 5.1 shows typical orientation of copepodids on ASSE culture surface during both burrowing and settled behaviour.

Measurement of Feeding by Cultured Copepodids

Figure 5.7 shows the effects of supplementing ASSE and seawater, for culture controls, with carmine red dye on copepodid survival and also observations of copepodid dye ingestion in both environments.

The addition of carmine red to the *in vitro* culture environment has no significant effect on copepodid survival either when incorporated into ASSE or added to seawater. When the dye was added to seawater containing copepodids the incidence of particles seen in the louse gut was very low with mean recorded levels of below 10% of the surviving population.

When copepodids were incubated with ASSE containing carmine red the first incidence of dye found in the gut was on day 2 (<10% population). The proportion of the louse population with ingested dye increased as the culture cycle progressed and peaked at days 7 and 8 where approximately 60% of the surviving population was observed with dye in the gut. Copepodids incubated in seawater, without a cellular substrate, showed dye ingestion rates significantly below those seen in animals maintained on ASSE. These differences, in conjunction with observations of the movement of dye through the gut in these individuals, were regarded as evidence of active feeding, rather than of passive uptake. After day 8 the proportion of feeding individuals within the population decreased until ingested dye particles were no longer seen beyond day 16 even though a surviving population was maintained in these experiments until day 20.

Figure 5.8 shows a copepodid removed from culture with ASSE containing carmine red, fixed in 10% buffered formal saline and viewed x100 magnification. Dark red dye is clearly visible in the centrally situated alimentary canal of the copepodid and accumulating towards the end of the gut. This later accumulation of red staining, along with areas located in the cephalic region

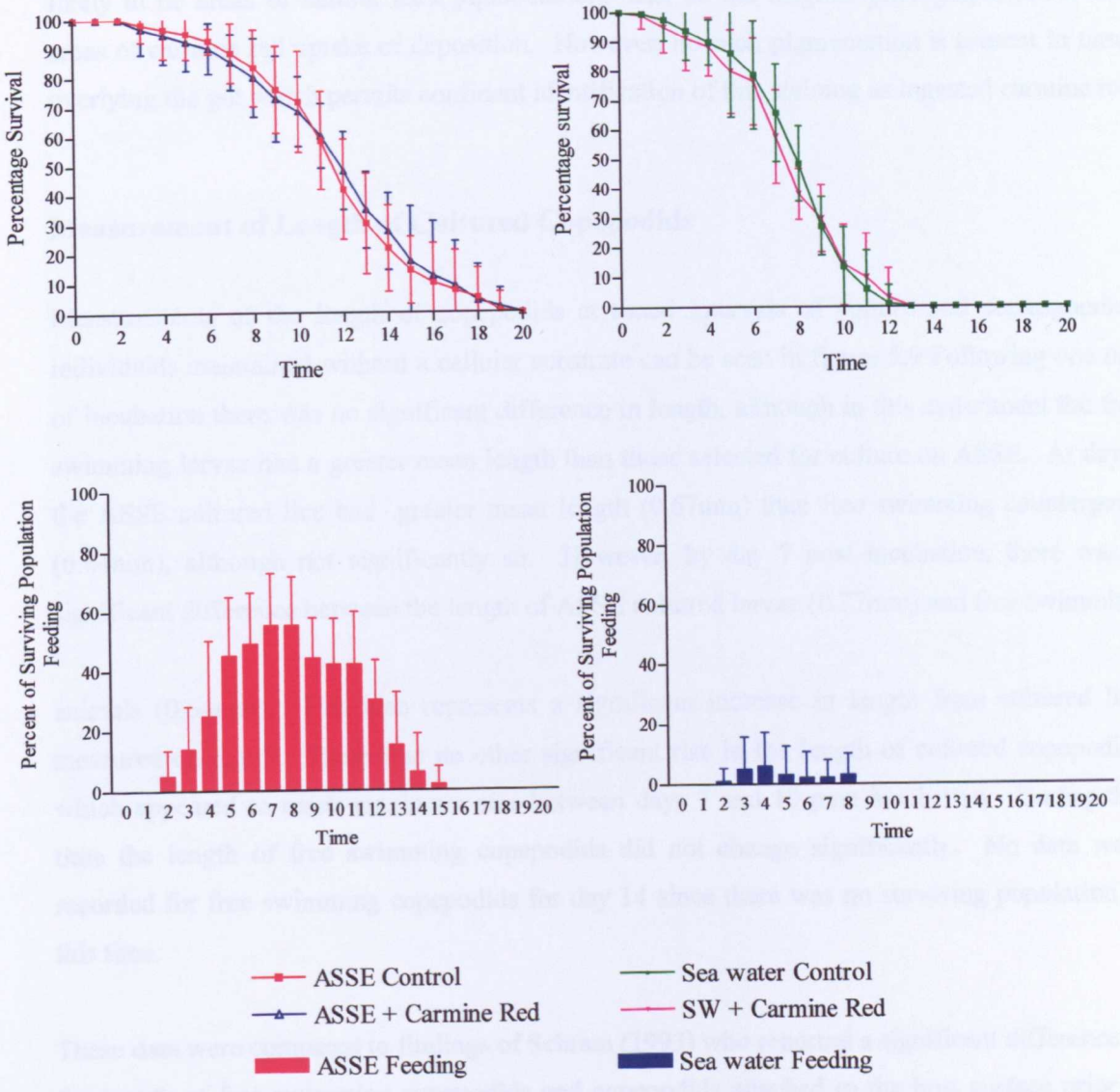


Figure 5.7

The survival and feeding of copepodids incubated with carmine red. The uppermost figures show the survival curves for copepodid populations maintained on ASSE and free swimming in seawater, with and without dye. The lower figures show the proportion of the surviving population observed with dye particles in the gut. n=12 per group

and genital segment were selected by digital processing and enhancement, but are thought more likely to be areas of natural dark pigmentation, seen on the original photograph, rather than areas of carmine red uptake or deposition. However, no such pigmentation is present in tissue overlying the gut which permits confident identification of this staining as ingested carmine red.

Measurement of Length of Cultured Copepodids

Measurements of the length of copepodids at timed intervals of culture and corresponding individuals maintained without a cellular substrate can be seen in figure 5.9 Following one day of incubation there was no significant difference in length, although in this experiment the free swimming larvae had a greater mean length than those selected for culture on ASSE. At day 3 the ASSE cultured lice had greater mean length (0.67mm) than free swimming counterparts (0.64mm), although not significantly so. However, by day 7 post incubation, there was a significant difference between the length of ASSE cultured larvae (0.77mm) and free swimming

animals (0.64mm). This also represents a significant increase in length from cultured lice measured on day 3. There was no other significant rise in the length of cultured copepodids which appeared to reach maximum size between days 7 and 10 post incubation. During this time the length of free swimming copepodids did not change significantly. No data were recorded for free swimming copepodids for day 14 since there was no surviving population at this time.

These data were compared to findings of Schram (1993) who reported a significant difference in the length of free swimming copepodids and copepodids attached to the host surface prior to moult to chalimus I. The author similarly found attached copepodids to have a greater length than free swimming individuals, and these data were not significantly different to those reported in this project.

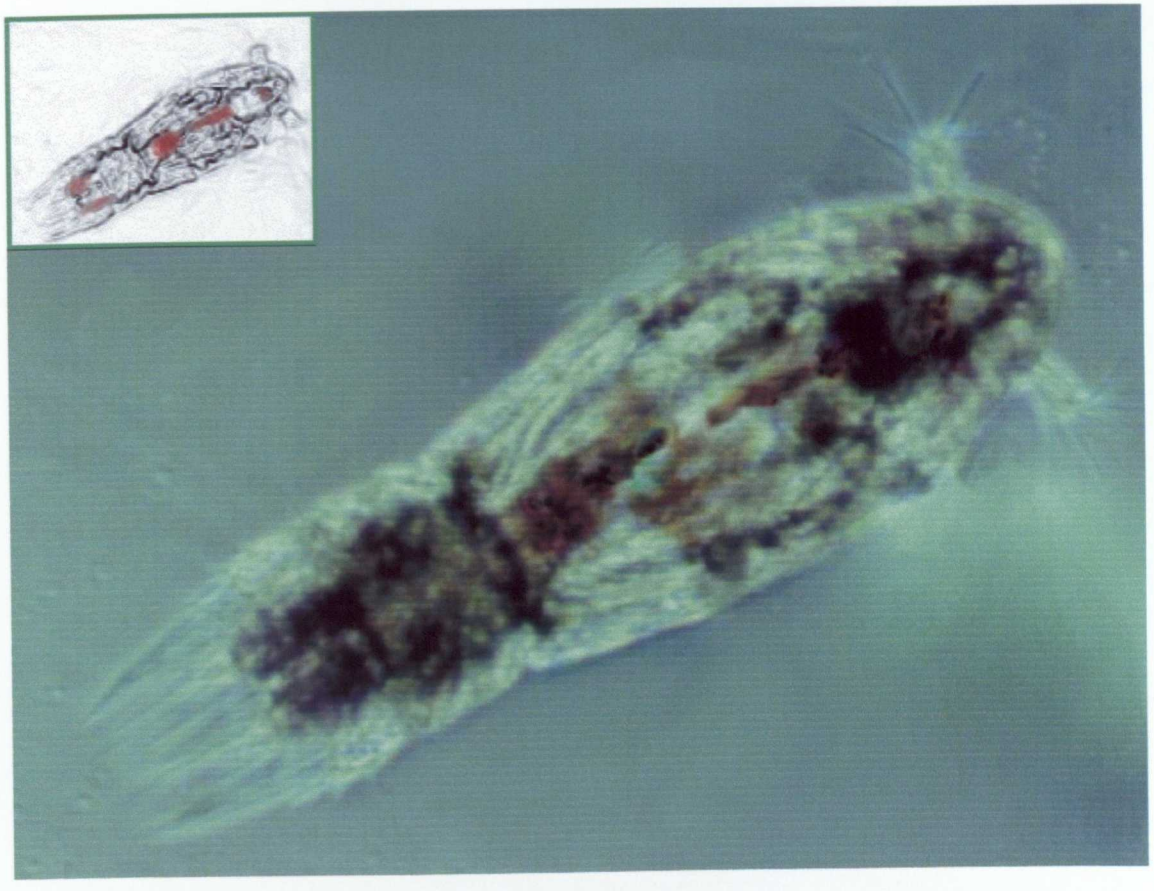


Figure 5.8

Copepodid (viewed x100) following incubation with ASSE supplemented with particles of carmine red dye. The inset picture is a digitally enhanced representation of the main figure modified to show the presence of dye in the gut following graphic colour calibration to select for the range of hue, colour and saturation of carmine red particles (Corel PhotoPaint version 8.0)

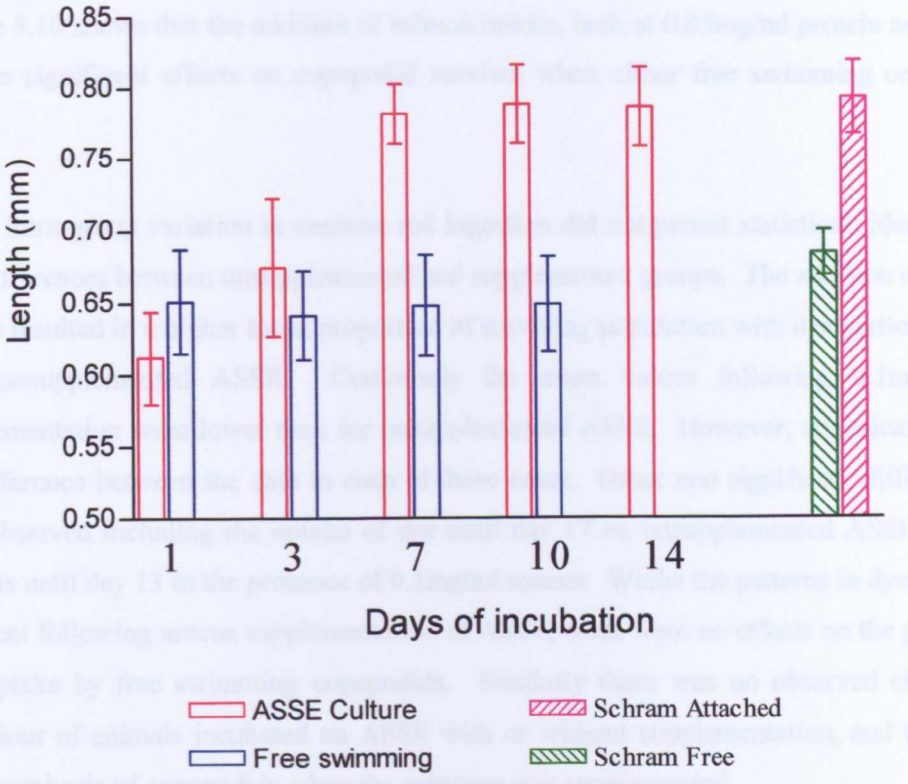


Figure 5.9

The measurement of the length of cultured copepodids. Data are mean length (± standard deviation) of 30 individuals measured at each time point both following incubation with ASSE and whilst free swimming without cellular substrate. The figure also contains data reported by Schram (1993) to allow direct comparison of results.

Measurement of Performance of Cultured Copepodids on ASSE Supplemented with Salmon Mucus

Figure 5.10 shows that the addition of salmon mucus, both at 0.05mg/ml protein and 0.1mg/ml, had no significant affects on copepodid survival when either free swimming or cultured on ASSE.

Large intra-group variation in carmine red ingestion did not permit statistical identification of any differences between unsupplemented and supplemented groups. The addition of 0.05mg/ml mucus resulted in a higher mean proportion of surviving population with dye particles in the gut than unsupplemented ASSE. Conversely the mean values following 0.1mg/ml mucus supplementation were lower than for unsupplemented ASSE. However, statistically there was no difference between the data in each of these cases. Other non significant differences were also observed including the uptake of dye until day 17 on unsupplemented ASSE which only persists until day 13 in the presence of 0.1mg/ml mucus. Whilst the patterns in dye uptake were different following mucus supplementation of ASSE, there were no effects on the proportion of dye uptake by free swimming copepodids. Similarly there was no observed change in the behaviour of animals incubated on ASSE with or without supplementation, and there was no metamorphosis of copepodids when the substrate was supplemented.

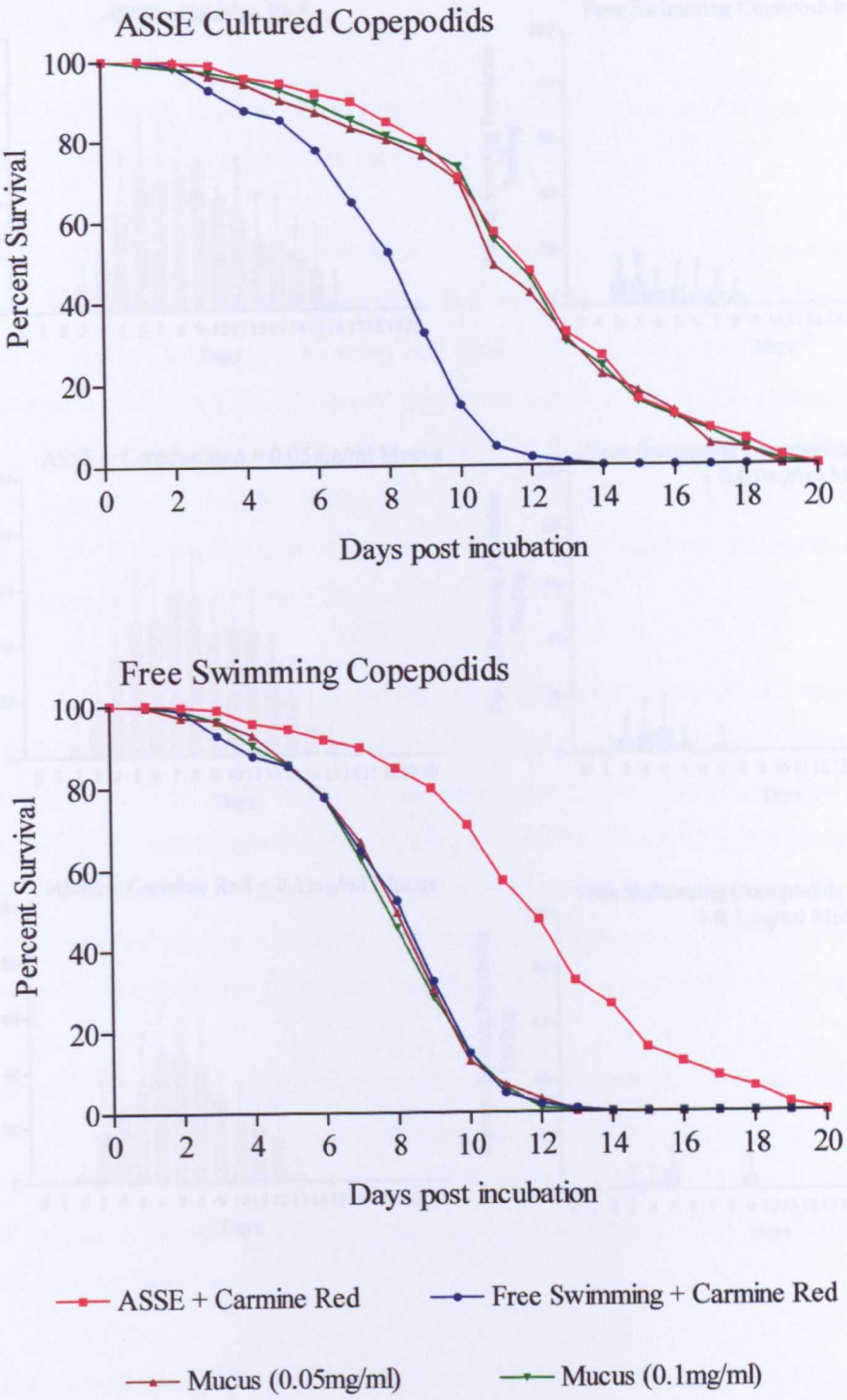


Figure 5.10

The survival of copepodids incubated with carmine red and salmon mucus supplemented ASSE and seawater. Data are mean of 12 observations for each group at each time point. A complete data set, including error bars can be seen in the appendices to this thesis.

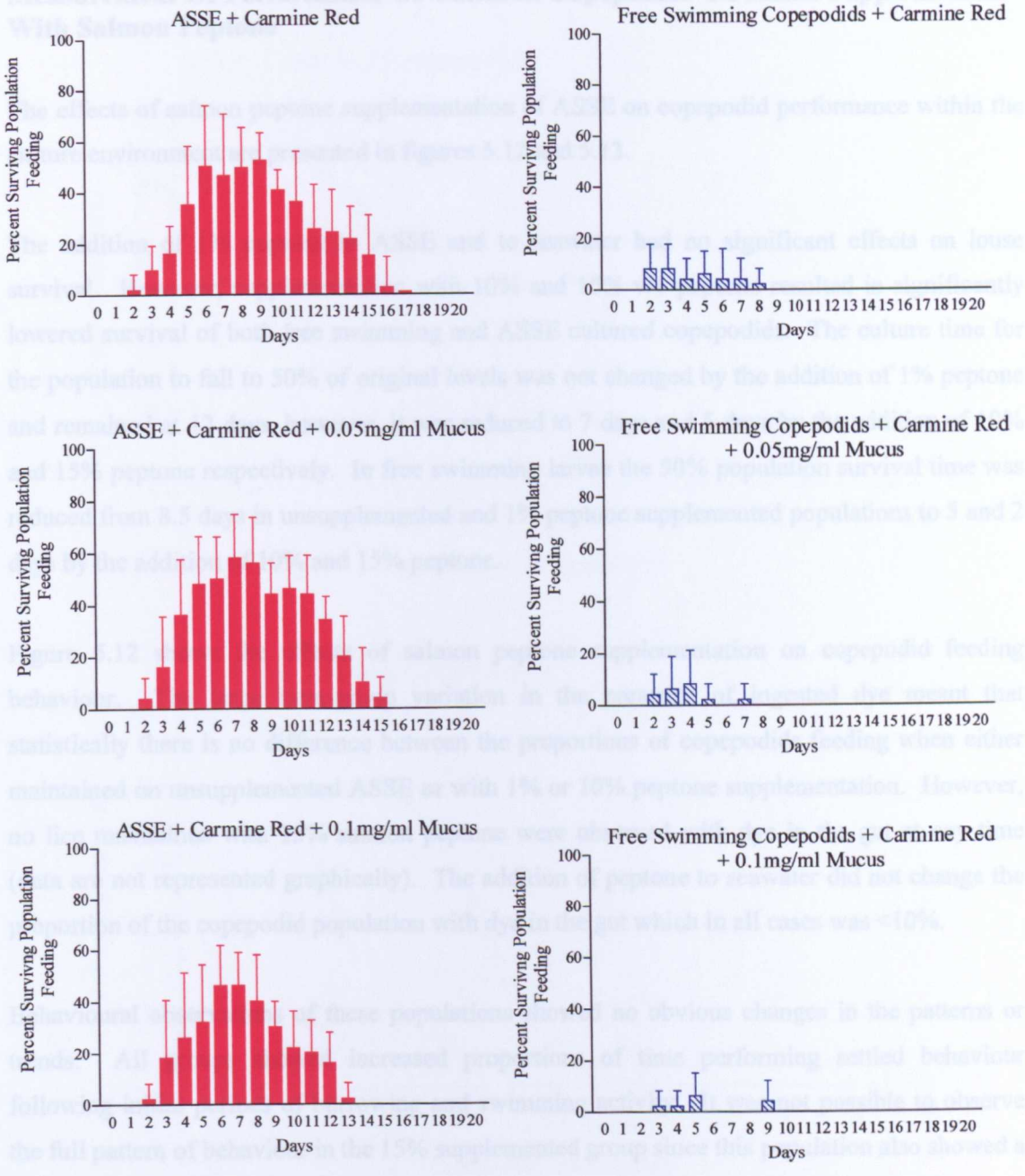


Figure 5.11

The feeding of copepodids incubated with carmine red dye and salmon mucus. Data are mean (± standard deviation) of 12 observations and show the proportion of the surviving copepodid population with dye particles in the gut. Copepodid populations were maintained with ASSE and controls were free swimming.

Measurement Of Performance Of Cultured Copepodids On ASSE Supplemented With Salmon Peptone

The effects of salmon peptone supplementation of ASSE on copepodid performance within the culture environment are presented in figures 5.12 and 5.13.

The addition of 1% peptone to ASSE and to seawater had no significant effects on louse survival. However, supplementation with 10% and 15% v/v peptone resulted in significantly lowered survival of both free swimming and ASSE cultured copepodids. The culture time for the population to fall to 50% of original levels was not changed by the addition of 1% peptone and remained at 12 days, however, it was reduced to 7 days and 5 days by the addition of 10% and 15% peptone respectively. In free swimming larvae the 50% population survival time was reduced from 8.5 days in unsupplemented and 1% peptone supplemented populations to 5 and 2 days by the addition of 10% and 15% peptone.

Figure 5.12 shows the effects of salmon peptone supplementation on copepodid feeding behaviour. The large intra-group variation in the presence of ingested dye meant that statistically there is no difference between the proportions of copepodids feeding when either maintained on unsupplemented ASSE or with 1% or 10% peptone supplementation. However, no lice maintained with 15% salmon peptone were observed with dye in the gut at any time (data are not represented graphically). The addition of peptone to seawater did not change the proportion of the copepodid population with dye in the gut which in all cases was <10%.

Behavioural observations of these populations showed no obvious changes in the patterns or trends. All groups showed increased proportions of time performing settled behaviour following initial periods of burrowing and swimming activity. It was not possible to observe the full pattern of behaviour in the 15% supplemented group since this population also showed a reduced survival rate.

Supplementation of ASSE by salmon peptone did not stimulate copepodid metamorphosis to chalimus and showed no improvements in terms of louse performance over the existing ASSE construction.

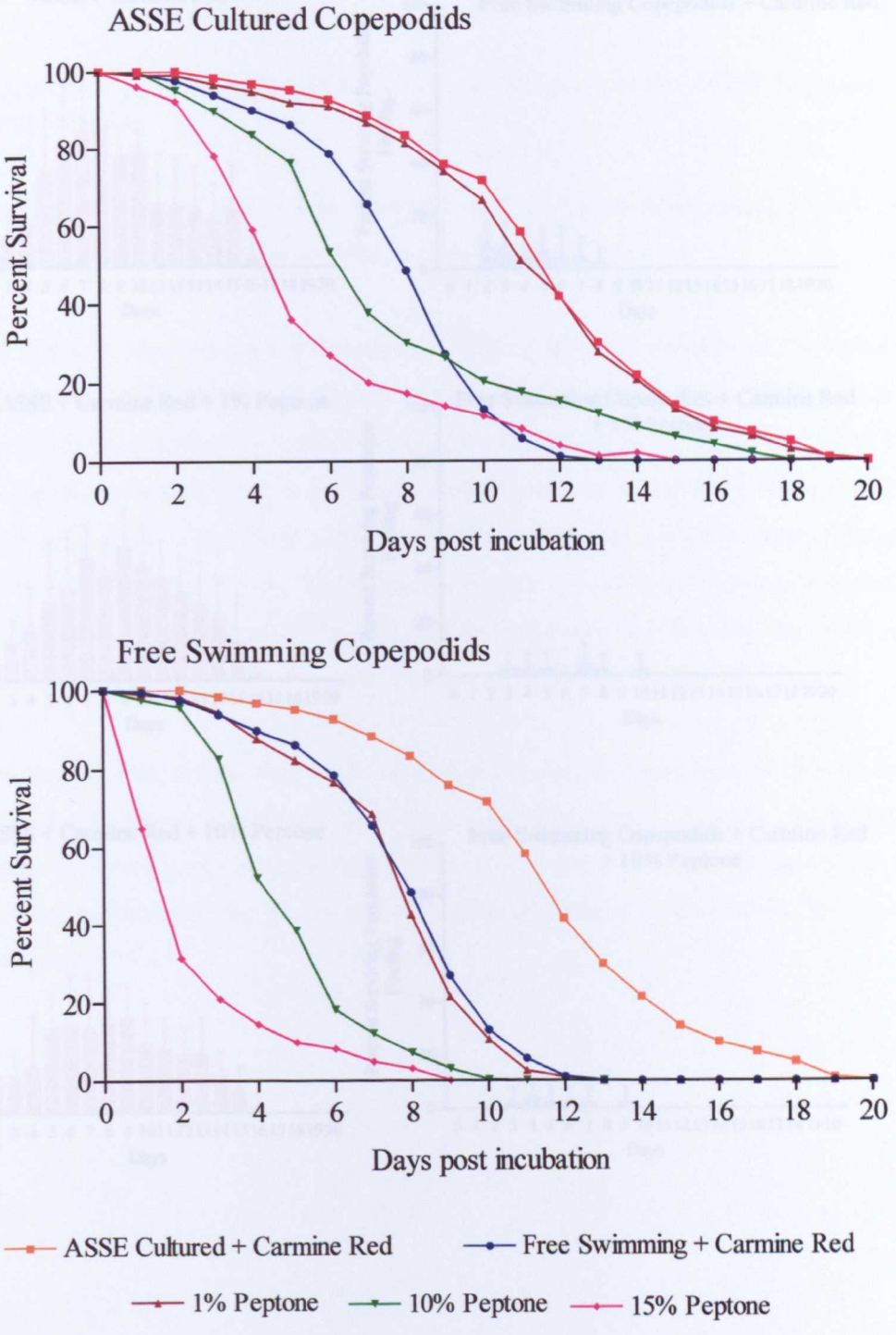


Figure 5.12

The survival of copepodids incubated with carmine red and salmon peptone supplemented ASSE and seawater. Data are mean of 12 observations for each group at each time point. A complete data set, including error bars can be seen in the appendices to this thesis.

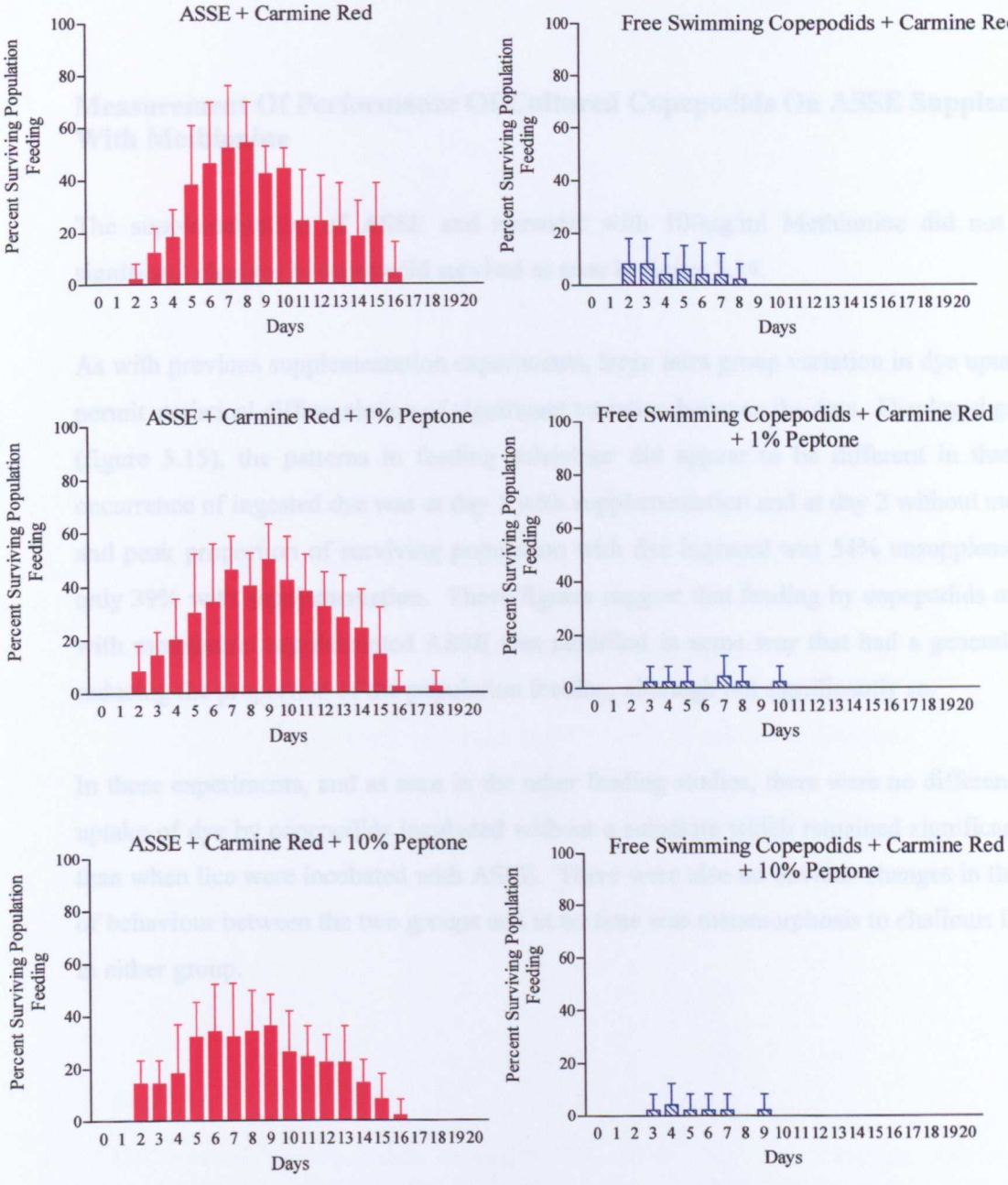


Figure 5.13

The feeding of copepodids incubated with carmine red dye and salmon peptone. Data are mean (± standard deviation) of 12 observations and show the proportion of the surviving copepodid population with dye particles in the gut. Copepodid populations were maintained with ASSE and controls were free swimming.

Measurement Of Performance Of Cultured Copepodids On ASSE Supplemented With Methionine

The supplementation of ASSE and seawater with 100μg/ml Methionine did not result in significant changes in copepodid survival as seen in figure 5.14.

As with previous supplementation experiments, large intra group variation in dye uptake do not permit statistical differentiation of significant variation between the data. Displayed graphically (figure 5.15), the patterns in feeding behaviour did appear to be different in that the first occurrence of ingested dye was at day 1 with supplementation and at day 2 without methionine, and peak proportion of surviving population with dye ingested was 54% unsupplemented and only 39% with supplementation. These figures suggest that feeding by copepodids maintained with methionine supplemented ASSE was modified in some way that had a general effect of reducing the proportion of the population feeding, although not significantly so.

In these experiments, and as seen in the other feeding studies, there were no differences in the uptake of dye by copepodids incubated without a substrate which remained significantly lower than when lice were incubated with ASSE. There were also no obvious changes in the patterns of behaviour between the two groups and at no time was metamorphosis to chalimus I observed in either group.

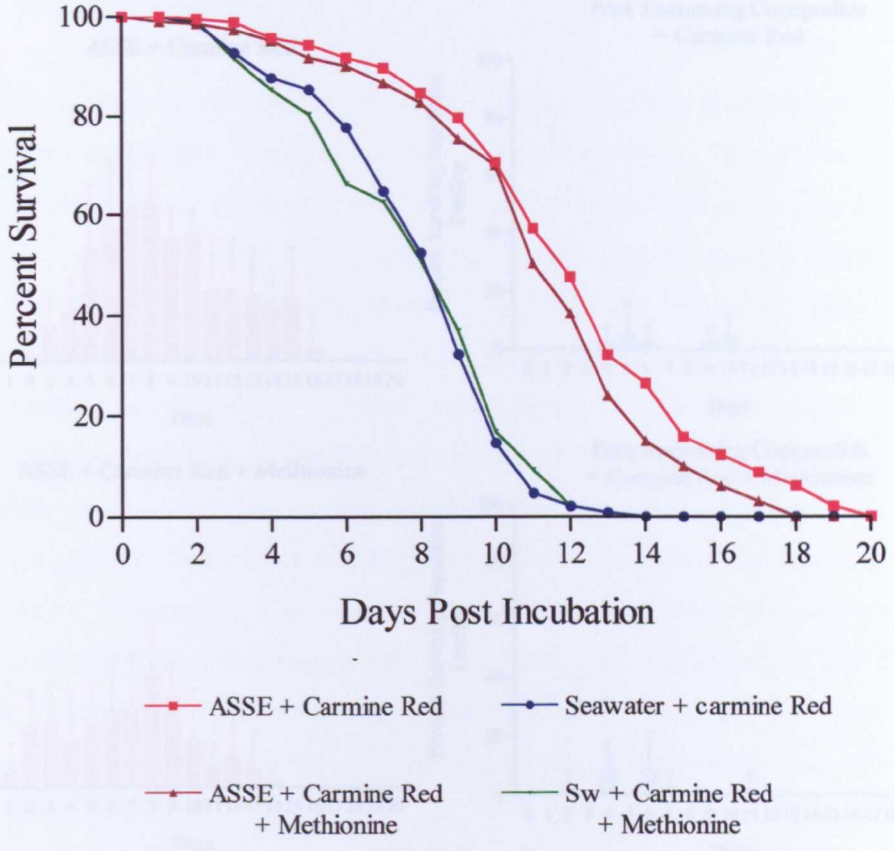


Figure 5.14

The survival of copepodids maintained on ASSE and free swimming following supplementation with 100µg/ml methionine. Data are mean percent survival from 12 observations at each time point. A complete data set, including error bars can be seen in the appendices to this thesis.

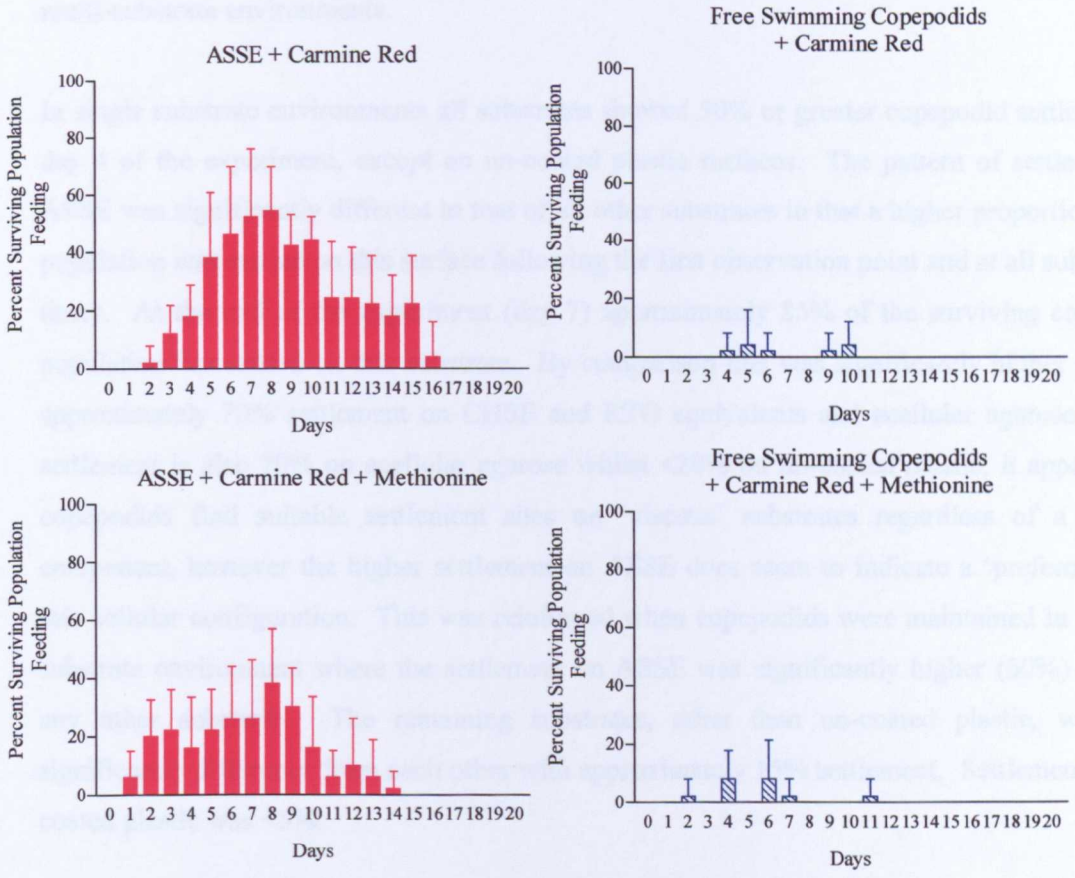


Figure 5.15

The observation of carmine red particles in the gut of copepodids incubated with ASSE and free swimming following supplementation with 100µg/ml methionine. Data are mean (± standard deviation) of the surviving population with ingested dye. n=12

Host Selection By Cultured Copepodids

Figure 5.16 shows the results of substrate settlement patterns of copepodids in both single and multi-substrate environments.

In single substrate environments all substrates showed 50% or greater copepodid settlement by day 4 of the experiment, except on un-coated plastic surfaces. The pattern of settlement on ASSE was significantly different to that of all other substrates in that a higher proportion of the population was settled on this surface following the first observation point and at all subsequent times. At the end of the experiment (day 7) approximately 85% of the surviving copepodid population has settled on this substrate. By comparison this was significantly higher than the approximately 70% settlement on CHSE and RTG equivalents and acellular agarose. Since settlement is also 70% on acellular agarose whilst <20% on un-coated plastic, it appears that copepodids find suitable settlement sites on 'viscous' substrates regardless of a cellular component, however the higher settlement on ASSE does seem to indicate a 'preference' for this cellular configuration. This was reinforced when copepodids were maintained in a multi-substrate environment where the settlement on ASSE was significantly higher (60%) than on any other substrate. The remaining substrates, other than un-coated plastic, were not significantly difference from each other with approximately 15% settlement. Settlement on un-coated plastic was <5%.

The supplementation of substrates with 0.1mg/ml mucus (figure 5.17) did not significantly effect the previous results in that settlement on ASSE was still approximately 60% at day 7 and significantly greater than the other substrates. However, with mucus supplementation the settlement rate on acellular agarose was significantly less (<5%) than the other cellular substrates (approximately 15%). Supplementation with 1% salmon peptone resulted in a different distribution pattern. Settlement on ASSE remained significantly higher than the other groups but at a level of only 45% by day 7. CHSE equivalent showed a significantly higher settlement rate (25%) than RTG equivalent (18%) which are both greater than the levels recorded when there was no substrate supplementation. Settlement on acellular agarose was significantly lower following peptone supplementation.

These experiments indicate that copepodids of *L. salmonis* are capable of settlement on a range of substrates in the absence of any alternative. However, when provided with that alternative the results suggest a preference for settlement on ASSE, containing Atlantic salmon cells. It also seems that following the addition of salmon peptone the settlement on non-Atlantic salmon

substrates increased which may indicate the presence of and response to a biochemical marker on ASSE that does not occur on the other salmonid cell lines used, but which may be present in salmon peptone.

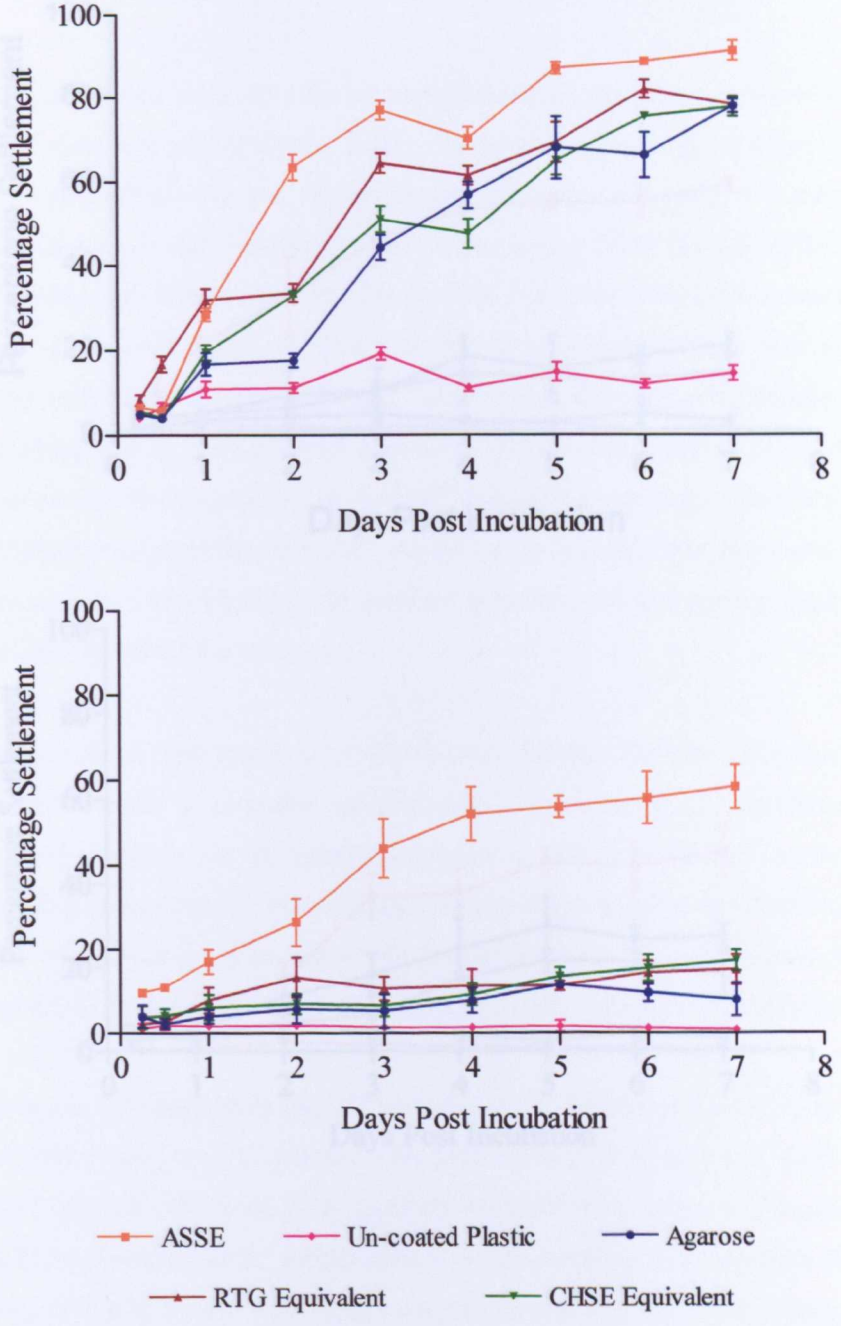


Figure 5.16

Substrate selection by cultured sops. The upper figure shows the pattern of settlement of copepodids on tissue culture substrates when maintained in a single substrate environment (no choice). The bottom figure shows the pattern of settlement when copepodids were maintained in an environment consisting of 5 different tissue culture substrates (choice). n=4, total number of copepodids observed is 480.

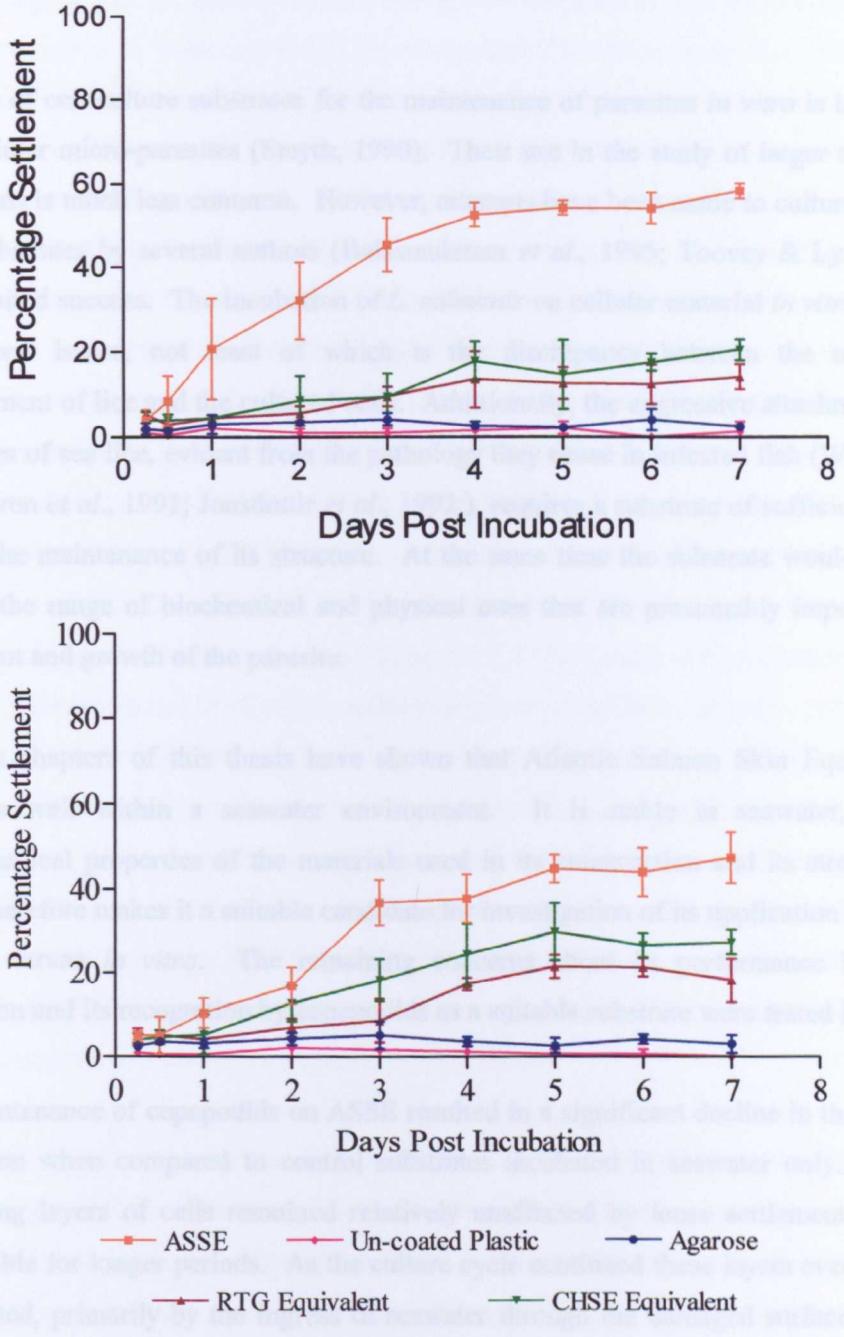


Figure 5.17

Substrate selection by cultured copepodids following supplementation of tissue culture substrates with 0.1mg/ml salmon mucus (upper figure) and 1% v/v salmon peptone. Data are mean number (± standard deviation) of copepodids in contact with each substrate in an environment consisting of equal surface area of 5 different substrates (choice), n=4, total number of copepodids observed 480.

DISCUSSION

The use of cell culture substrates for the maintenance of parasites *in vitro* is largely limited to intracellular micro-parasites (Smyth, 1990). Their use in the study of larger and ectoparasitic organisms is much less common. However, attempts have been made to culture *L. salmonis* on such substrates by several authors (Balasundaram *et al.*, 1995; Toovey & Lyndon, 2000) but with limited success. The incubation of *L. salmonis* on cellular material *in vitro* is complicated by several issues, not least of which is the discrepancy between the optimum culture environment of lice and the cultured cells. Additionally, the aggressive attachment and feeding processes of sea lice, evident from the pathology they cause in infected fish (Wootten & Smith, 1980; Bron *et al.*, 1991; Jonsdottir *et al.*, 1992;), requires a substrate of sufficient robustness to enable the maintenance of its structure. At the same time the substrate would be required to display the range of biochemical and physical cues that are presumably important in normal settlement and growth of the parasite.

Previous chapters of this thesis have shown that Atlantic Salmon Skin Equivalent (ASSE) performs well within a seawater environment. It is stable in seawater, owing to the biomechanical properties of the materials used in its construction and its stratified structure, which therefore makes it a suitable candidate for investigation of its application in the culture of sea lice larvae *in vitro*. The remaining concerns about its performance following louse incubation and its recognition by copepodids as a suitable substrate were tested in this chapter.

The maintenance of copepodids on ASSE resulted in a significant decline in the substrates cell population when compared to control substrates incubated in seawater only. However, the underlying layers of cells remained relatively unaffected by louse settlement behaviour and were viable for longer periods. As the culture cycle continued these layers eventually began to be affected, primarily by the ingress of seawater through the damaged surface layers and not because of copepodid behaviour, since animals appeared to remain relatively inactive following the initial settlement process. The structure remained intact for approximately 10-14 days before all cellular organisation was lost. However, some small cell aggregations did still remain which were capable of supporting continued louse settlement. The robustness of the substrate is therefore questionable in its present form, since it was not capable of sustaining sea lice throughout their life cycle, although is was capable of maintenance of copepodids for periods significantly greater than would be expected in the absence of a cellular substrate.

However, copepodid settlement on the substrate indicates a physiological recognition of it as an appropriate surface for attachment and this could be argued to be a more important result than the actual period of louse survival. The recognition of suitable hosts is a vital process in host finding by all parasites that typically involves both behavioural and biochemical mechanisms (Cox, 1993). Bron *et al.* (1991) described the settlement and attachment processes of *L. salmonis* copepodids as occurring in 3 phases; searching phase; primary attachment phase; and secondary filament attachment stage. In later work (Bron *et al.*, 1993), the authors tested copepodid responses to physical and biochemical stimuli from which they proposed the events involved in host detection. Copepodid larvae are positively phototaxic and also move towards areas of low pressure which, in the natural environment, would bring them into surface water where they might be better positioned to come into contact with hosts. They do not appear to respond to chemical host factors, at least not at distance, but are capable of detecting vibration and movement (Bron, *et al.*, 1993; Heuch & Karlson, 1998). The reception of these stimuli elicit 'burst swimming' and 'looping' behaviour along with active 'grappling' of passing fish. Upon attachment to the potential host, Bron *et al.* (1993) suggest that chemoreceptors located on the antennules make the final chemical determination of its suitability. The involvement of these structures in host recognition is also suggested by Hull *et al.* (1998) who observed the reduced ability of male sea lice to resettle on host fish following the ablation of setae on the distal segment of the antennules, whilst uninjured animals relocated successfully. Recognition of a suitable host by copepodids stimulates attachment and metamorphosis, whilst identification of an unsuitable substrate will cause the copepodid to detach and re-enter the water column (Bron, *et al.*, 1991; Bron, *et al.*, 1993). The exact nature of the chemical cue or the specific surface receptors and markers involved are not known.

The standard *in vitro* environment of the culture experiments performed in this study provides only a single substrate choice. Therefore the settlement of copepodids on ASSE can not automatically be taken as a sign of its suitability, especially given that the study also showed copepodid settlement on other non-Atlantic salmon and non-cellular substrates. However, the provision of multiple substrates in 'choice chamber' experiments suggested a preference for Atlantic salmon based ASSE. In these experiments the majority of copepodids settled on ASSE with significantly fewer choosing to settle on rainbow trout and chinook salmon cell based substrates. The reasons for the obvious 'choice' to settle on ASSE are evidently connected to either the biochemistry of Atlantic salmon cells, the production of Atlantic salmon specific semiochemicals or to specific cell surface receptors/topography that they possess and continue to express in the culture environment. Digenean parasites, such as Schistosomes are known to identify their hosts based on their detection of profiles of specific fatty acids secreted from host

skin which can be mimicked *in vitro* to encourage their penetration of artificial and non-host substrates (Haas *et al.*, 1987; Haas *et al.*, 1991; Fusco *et al.*, 1993; Haas *et al.*, 1995). Similarly, Whittington *et al.* (2000) suggest that the recognition of fish hosts by monogenean parasites is based on the positive reaction between species specific mucus components of the host and secretions from the parasite's anterior adhesive areas that form a cement between the two animals. This mechanism does not function on non-niche hosts but is still considered a temporary attachment process that allows more comprehensive identification of the host by the recognition of chemicals or surface structures on the host epidermis, the exact nature of which is still unclear. Whilst the mechanisms involved in *L. salmonis* copepodids choosing to settle on ASSE are not known, their settlement on the substrate is a testament of its suitability to their maintenance *in vitro* and its applicability to their study in this environment.

Smyth (1990) concluded from a series of *in vitro* culture experiments of helminth parasites that they were tolerant of a wide range of biochemical and environmental factors that did not affect their survival, metamorphosis and development *in vitro*. However, Frank and Weinstein (1984) reported that the helminth parasite *Dipetalonnena viteae* had very specific culture requirements that, if not met, did not allow normal development. The assessment of the normality of cultured organisms is obviously a pivotal step in determining that a culture environment and process are suitable. This present study assessed normality of cultured *L. salmonis* in terms of copepodid survival, behaviour, metamorphosis, growth and feeding.

Experiments and observations concluded that copepodids incubated with ASSE showed increased survival rates when compared to those maintained on excised salmon skin, rainbow trout and chinook salmon cellular substrates, and non-cellular substrates. Typically, copepodids maintained *in vitro* without substrate persist for approximately 10-12 days (Dawson *et al.*, 1997; Johnson & Albright, 1991; Johnson & Albright, 1992) which was also the case in experiments performed in the current study. In the presence of ASSE the maximum recorded survival time was 19 days with a 50% population survival period of 12 days. However, in the natural environment and in experimental infection studies copepodids persist for not normally more than 3-4 days before metamorphosis to chalimus I (Johnson and Albright, 1992; Dawson, et al., 1997; Pike & Wadsworth, 1999). Copepodids cultured on ASSE did not metamorphose to chalimus stages. Development to these stages of the parasite life cycle would have represented a significant milestone for the project and for the validation of ASSE as a suitable sea louse culture model. However, since metamorphosis did not occur it was important to identify the processes of normal development that cultured copepodids did go through. In doing this an explanation for the lack of metamorphosis may have presented itself and led to the development

of methods that could encourage its occurrence. Furthermore, this type of investigation would assist in the validation of the ASSE substrate as a louse culture surface, if only one that in its present form was capable of only the extended maintenance of the copepodid stage.

Work by Schram (1993) identified an increase in the size of copepodids attached to hosts before metamorphosis to chalimus I when compared to free-swimming individuals. Measurements from this present study showed a significant increase in the size of copepodids settled on ASSE when compared to those incubated without a substrate, that were broadly of the magnitude of difference reported by Schram (1993). The assumption is made that upon identification of a suitable host the mechanisms of metamorphosis, including changes in physiology, biochemistry and anatomy are stimulated. Evidence is limited with regard to the specific processes occurring during the pre-moult and moult of *L. salmonis* larvae. In arthropods, cuticle does not normally expand and so growth only occurs following the moult when new, soft cuticle is produced and expanded. Consequently growth occurs in a series of steps. However, in regions of membranous cuticle, or in animals that posses a wholly membranous cuticle, growth may be continuous and limited only by the elasticity of the cuticle. The extent of membranous cuticle in the integument of *L. salmonis* copepodids is not known. Adults of the marine ectoparasitic copepod *Lernaeocera branchialis* undergo a process of elongation following the final moult (Smith & Whitfield, 1987). The mechanism of elongation consists of a straightening out of a highly folded abdominal cuticle. This is evident beneath the cuticle of the chalimus stages and can generate an approximately 6-fold increase in length. However, similar structures have not been observed in the cuticle of *L. salmonis*. A more likely explanation is a low calcium content producing a relatively flexible cuticle that may allow expansion by deformation of the cuticle to a limited extent (*pers. comm.* Dr. James Bron, University of Stirling, Scotland). However, even though the exact mechanism is unclear, the hypothesis is suggested that the recorded increase in size of cultured copepodids is connected to their preparation for moulting.

Free-swimming copepodids possess a functional gut and external mouth parts but remain non-feeding until they settle on a host (reviewed by Pike and Wadsworth, 1999), and in due course moult to chalimus I. In the present study copepodids that were settled on ASSE were observed to be feeding, which was measured by the uptake and presence in the gut of dye particles included in the substrate construction. The on-set of feeding corresponds to the timing of final settlement of the larvae on the substrate from around day 3 of incubation onwards, where previous time was spent performing periods of swimming and active burrowing behaviour. Following 7 days of incubation more than 50% of the copepodid population maintained on

ASSE were observed feeding. A nutritional involvement in the stimulation of moulting cannot be ruled out. It may be the case that whilst feeding and growth of copepodids is observed, indicating adequate nutrition, the energetics and biochemical nature of the food source may be incorrect. Calorifically, they appear receive sufficient nutrition to sustain them, but inappropriate balances between nutritional components, such as the protein:lipid ratio important in the development of Schistosomes (Fusco *et al*, 1993), or the absence of essential amino and fatty acids or vitamin groups, may not stimulate metamorphosis, or provide the appropriate precursors of important hormones or steroids required for metamorphosis to occur. Alternatively, ASSE may lack sufficient quantities of a particular compound or nutritional group that might require accumulation above a threshold level to provide the correct cue for the animals' metabolism to commit to metamorphosis.

The identification of the specific chemical and/or nutritional components that may be required to stimulate metamorphosis is a prohibitively extensive process that could not be thoroughly achieved as part of this project. As a compromise, Atlantic salmon mucus and peptone were incorporated into the ASSE structure to provide broad-spectrum nutritional additives. In addition, DL-methionine was used as a more specific nutritional component with well researched involvement in the synthesis of insect growth and moulting hormones (Wainwright *et al.*, 1996). The addition of mucus, methionine and, at low levels, salmon peptone did not affect either the survival, behaviour or feeding of copepodids incubated on ASSE. The expectation was that the inclusion of mucus, which is ingested by larval lice attached to host fish, and which is involved in the chemical recognition of hosts by other fish parasites (Whittington, *et al.*, 2000), might modulate both the settlement rates and feeding dynamics of cultured lice. Its lack of influence indicates that the unsupplemented ASSE provides the required cues to encourage louse settlement, although not metamorphosis. That metamorphosis is not stimulated by salmon mucus suggests that the 'missing trigger' is one that, during a normal infection, would be received after settlement on the host and not during host recognition or necessarily during the settlement process. Provision of these absent cues hoped to be proved by the addition of salmon peptone, but this also did not result in significant changes to settlement, survival or feeding, except at higher concentrations where survival of lice on ASSE was significantly reduced. Fish peptone provides a broad range of amino acid, peptides, lipids, hormones and metabolic precursor substances and is used as a nutritional supplement of marine bacteria in culture (Page & Cornish, 1993), and was used as a medium in the early study of cultured animal cells (Lewis, 1916). However, in this present application it did not provide the appropriate chemical or nutritional cues required for copepodid metamorphosis. Different

results may be achieved using peptone produced by proteolytic digestion that might conserve the structure of tissue components more effectively than the heating method used in this study which undoubtedly caused extensive structural biochemical degradation.

The exclusion of methionine from the media supporting endocrine tissue of *Cancer pagarus in vitro* prevents the release of moulting hormones by the tissue (Yudin *et al.*, 1980), and is known to be limiting in the normal growth and development of other crustaceans (Yudin, et al., 1980; Wainwright, et al., 1996) and insects (Telfer & Kunkel, 1991). However, its inclusion in ASSE did not stimulate moulting and metamorphosis. It may be the case that this amino acid is not required by *L. salmonis* copepodids during their development and/or metamorphosis. It may also be that this, and the other culture supplements added to ASSE were delivered at the wrong time to stimulate the response they might have had *in vivo*. The provision of large concentrations of mixed chemicals consistently throughout the culture process may have overwhelmed the receptor sites for the developmental cues, or competed for access to these sites which produced metabolic changes other than metamorphosis. Further development of the ASSE should focus on delivering supplementing chemical in controlled doses at specific points during culture, and even during naupliar development since the correct cues for subsequent metamorphoses may be received from exogenous sources early in the animals' life cycle.

This study has assessed the suitability of ASSE as a tissue culture substrate for the culture of *L. salmonis in vitro*. Attempts at modifying its structure and chemistry did not result in the improved performance of the cultured animals. These remained able to survive for extended periods at the copepodid stage, feed, grow and perform the normal range of settlement behaviours. The eventual identification of the absent developmental cues, whether they are physical or chemical, may lead to copepodid development to chalimus I, as might the provision of exogenous arthropod moulting and growth hormones. However, in its present form ASSE still remains a valuable tool that can be used to investigate the interactions between host and the infective parasite stage as well as possibly providing a medium for the analysis of the effects of medicines on this stage. Many authors have recognised the importance of preventing parasite infection and larval recruitment to break the infection cycle (Wadsworth *et al.*, 1998; Pike and Wadsworth, 1999) and in this respect ASSE could be incredibly important. Furthermore, the copepodid represents the parasite stage that is first in contact with the host and as such is vulnerable to the full weight of its responses to infection. To be in possession of a method that permits the apparently normal association of parasite and host tissue for artificially extended periods is a great advantage in understanding the mechanisms involved during early attachment that permit successful parasitism.

The Effects of Culture Supernatants on Salmon Macrophage Immune Functioning

Chapter 6

INTRODUCTION

THE EFFECTS OF CULTURE SUPERNATANTS ON SALMON MACROPHAGE IMMUNE FUNCTIONING

The function and form of fish macrophages are reviewed by Secombes & Fletcher, 1992). Macrophages are large agranular phagocytic cells distributed throughout the body tissues of teleosts, including the blood, the peritoneal cavity and lymphoid system, especially the kidney. Their isolation from the latter provides the largest number of cells; commonly around $2x10^7$ cells/ml (Secombes, 1990); these cells are adherent to glass and plastic culture surfaces and are sustainable for extended periods which make them a very practical tissue for *in vitro* study (Secombes, 1990). They are known to maintain their *in vivo* functions during laboratory maintenance and so have been used to measure both their normal functional characteristics such as phagocytosis, chemotaxis, bactericidal activity and antigen processing and presentation (Janeway *et al.*, 1999), as well as the effect on normal functions of exogenous chemicals, pathogens and environmental changes (Weeks *et al.*, 1986; Secombes *et al.*, 1991; Secombes & Fletcher, 1992). The correlation of *in vitro* and *in vivo* macrophage responses and the pivotal involvement of these cells in the 'whole organism response' means that macrophage assays are used extensively as indicators both of the systemic outcome to, for instance pathogenic invasion, and of the health status of an individual or population (Paulnock, 2000).

Macrophages form the second phase of a biphasic cellular response during inflammation, arriving at the site normally after granulocytes. They are attracted to the site of infection by both pathogen and host derived chemoattractants, the latter including complement components and eicosanoids (Secombes and Fletcher, 1992). Eicosanoids are a group of lipid mediators derived from membrane phospholipids via the action of, initially, phospholipases and then either cyclooxygenases to produce prostaglandins, or lipoxygenases to produce leukotrienes and lipoxins (Rowley, 1991; Rowley, 1996). The exact biochemical/physical processes that occur during chemoattraction of fish leucocytes *in vivo* is not fully understood but is likely to follow the events sequence seen in mammals and other higher vertebrates. The process has 3 phases, the first being that of un-stimulated leucocytes moving through the vasculature, followed by activation upon the reception of chemoattractant products that causes the affinity between leucocyte and endothelial cell integrins and glycoproteins to increase and fixes the cell to the vessel wall. The final stage is extravasation where the cell migrates through the vessel wall

along the direction of the chemoattractant gradient toward the site of infection (Janeway, *et al.*, 1999; Paulnock, 2000). The ability of macrophages to detect and to move towards the attractant is a vital step in the cellular immune response. Laudan *et al.* (1986) first suggested the use of immunosuppressive prostaglandins by the microsporidian parasite *Glugea stephani* infecting winter flounder, *Pseudopleutronectes americanus*. In their experiments they showed that following the administration of indomethacin, a cyclooxygenase inhibitor, the immunosuppressive effects of the parasite could be reversed. Interference with the cellular immune response before it has a chance to develop, i.e. during chemotaxic signalling and cell recruitment, is an effective strategy employed by many parasites (Cox, 1984; Wikel *et al.*, 1994; Wikel *et al.*, 1996). By acting in this way they prevent their contact with macrophages without having to provide defences against the direct immunological functions of these cells such as phagocytosis and the release of oxygen derivatives.

Macrophages are phagocytic cells that upon arrival at the site of infection attempt to ingest pathogenic material. The process of phagocytosis has several stages, the first being chemotaxis and the movement of the cell toward the source of the attractant. This is followed by adherence, either between glycoproteins on the pathogen/macrophage surface, or via opsonins such as the complement component C3 which binds the organism and the C3 receptor of the macrophage (Secombes and Fletcher, 1992). Cytoplasmic evaginations form pseudopodia that surround the organism to create intracellular vesicles termed phagosomes that fuse with lysosomes resulting in the enzymatic digestion of the organism. Rowley *et al.* (1988) review the ability of fish phagocytes to destroy micro-organisms using lysosomal enzymes, however, they also possess oxidative mechanisms which have an equally efficient killing ability that can operate both intra- and extra-cellularly.

Fish phagocytes are able to produce Reactive Oxygen Species (ROS) generated through the action of a membrane bound enzyme equivalent to the NADPH oxidase of mammalian phagocytes (Secombes and Fletcher, 1992) during a process termed the respiratory burst. In this reaction molecular oxygen is reduced to superoxide anion which is then converted into a number of more potent bactericidal agents such as hydrogen peroxide, singlet oxygen and hydroxyl radicals (Secombes and Fletcher, 1992). In addition, hydrogen peroxide can interact with peroxidase, present in granulocytes, to generate hypohalites such as hypochlorite and chloramine, which are also potent bactericidal agents (Secombes and Fletcher, 1992).

The respiratory burst of fish phagocytes is evoked by various stimuli including particulate stimulants such as zymosan and bacteria, and complement opsonised foreign material

(Secombes and Fletcher, 1992). Additionally, soluble stimulants such as phorbol myristate acetate (PMA) can activate the oxidative process and have been used in standard *in vitro* assays to measure the function in isolated cells. (Chung & Secombes, 1987; Secombes, 1990; Secombes and Fletcher, 1992). Interference with oxidative defences of macrophages is a mechanism used by many intracellular parasites such as *Trypanosoma cruzi* (Cox, 1984; Cox, 1993; Liew & Cox, 1998), and arthropod parasites (Wikel, *et al.*, 1994; Wikel, *et al.*, 1996) to allow successful parasitism.

In addition to phagocytosis and the secretion of ROS, macrophages also secrete a number of other immunologically important molecules, notably cytokines and eicosanoids (Secombes and Fletcher, 1992). These have vast and varied effects within host tissues and as well as acting as chemoattractants for other phagocytic cells, as mentioned briefly earlier, they also exhibit direct effects upon the invading pathogen and act to augment or control on going biochemical reactions and the functioning of immune effector cells (Secombes and Fletcher, 1992; Sharp *et al.*, 1992; Sharp *et al.*, 1991; Whyte *et al.*, 1989). Both interleukin-1 (IL-1) –like (Clem *et al.*, 1991) and TNFα-like (Zeilkoff *et al.*, 1991) molecules have been seen to be released by fish macrophages, and it has been known for several years that fish leucocytes can be stimulated to release eicosanoids following incubation with calcium ionophore A23187 (Secombes and Fletcher, 1992). In mammalian systems the biochemical pathways and ultimate function of these substances are better understood than in fish, however their metabolic effects are well documented, as are the effects of their manipulation by parasitic organisms (Cox, 1984). Arthropod parasites such as the ixoid tick *Dermacentor andersoni* are known to suppress the production of IL-1 and TNF by macrophages of their murine hosts (Ramachandra & Wikel, 1992), as are *Ixodes ricinus* infecting rabbits (Schorderet & Brossard, 1994). Such suppression has been seen to impair T-lymphocyte activation, neutrophil activation, chemotaxis, Ig synthesis and anti-viral responses of the host and permit successful parasite infection.

Macrophages provide an effective and convenient *in vitro* model since, as has been discussed, they are a central component of the host immune defences, and, coincidentally, are capable of extended maintenance and manipulation *in vitro*. In this way they have been proven useful for the investigation of host responses to infection as well as pathogen modulation of the immune system during an infection. In the study of *L. salmonis* infections of Atlantic salmon, macrophages have been most recently, and notably, used by Mustafa *et al.* (2000). In this work cells were isolated from the head kidney of infected and uninfected fish and used in standard *in vitro* assays to measure their phagocytic and oxidative defence capabilities. These authors showed that the functional capabilities of head kidney macrophages did not change during the

infection; they were neither stimulated to respond nor suppressed by the attached larvae. However, by day 21 of the infection, at a time when pre-adult and early adult lice were appearing, the levels of blood glucose and cortisol, both indicators of chronic stress, had increased. This coincided with a significant reduction in macrophage phagocytosis and respiratory burst activity. By extrapolating the *in vitro* macrophage responses the authors concluded that *L. salmonis* did not interfere with the host systemic immune responses directly as had been previously suggested by Johnson and Albright (1991). They were not able to comment on the likelihood of localised immunodeppression at the site of parasite attachment however, which was also suggested by Johnson and Albright (1991). They reason that any observed system wide immunomodulation during *L. salmonis* infections is due to the physical manifestations of chronic stress, itself brought about by the physical insult on the host skin by the parasite and subsequent failure of osmoregulatory capabilities. The primary evidence for these conclusions is derived from the use of isolated macrophages in *in vitro* tests, which has become an accepted methodology in the prediction of whole animal responses in many animal phyla (Paulnock, 2000), including fish (Secombes, 1990; Secombes and Fletcher, 1992; Sharp, *et al.*, 1991; Whyte, *et al.*, 1989; Whyte, *et al.*, 1990).

The present study has already presented data on the effects of *L. salmonis* infection on Atlantic salmon macrophage functioning in chapter 3. This current chapter will employ them in a different way, to test their ability to perform their normal non-specific defence functions *in vitro* following incubation with *L. salmonis* culture products collected from experiments discussed in chapter 5. The *in vitro* response of macrophages to chemical treatments and the subsequent effects on their functioning has been used by many researchers (Paulnock, 2000). In fish this technique has been used extensively in both the study of the immunological effects of potential toxic compounds (Secombes, *et al.*, 1991), and of macrophage derived lipid mediators of the immune response; lymphokines (Chung and Secombes, 1987; Secombes, 1990; Secombes and Fletcher, 1992; Sharp, *et al.*, 1992). In these latter experiments, macrophages were stimulated to produce Macrophage Activating Factor (MAF) by the addition of concanavalin A and PMA. Populations of naïve salmon macrophages were then incubated with MAF containing supernatants and their effects on their functioning measured (Secombes, 1990). The methodology used in this chapter is comparable to that used by the above mentioned authors.

MATERIALS AND METHODS

Chapter 6 assesses the effects on macrophage functioning *in vitro* of incubation with sea louse larvae culture supernatants. Culture supernatants (Louse Culture Supernatant (LCS), Louse Control (LC) and Matrix Control (MC)), collected from experiments detailed in chapter 5 were incubated with naïve Atlantic salmon macrophages (isolated according to the method in section 2.8) for 24 hours using the method described in section 2.31.

The duration of incubation, and the concentration of supernatant used during the incubation were first determined by measuring the effects of varying concentrations on macrophage morphology and adherence characteristics, used as indicators of normality of the treated cells (section 2.32). Following optimisation and incubation with supernatants, the cells were then used in standard assays to measure their phagocytic (section 2.9), respiratory burst (section 2.10) and chemotaxic (section 2.11) responses. Each assay was performed using supernatant treated and untreated macrophages, and statistical differences between groups was determined using one way analysis of variance and paired two-sampled t-test where appropriate.

RESULTS

THE EFFECTS OF CULTURE SUPERNATANTS ON SALMON MACROPHAGE IMMUNE FUNCTIONING

Effects Of Supernatant Incubation On Macrophage Morphology And Adherence Properties

The normality of salmon macrophages following incubation with culture supernatants was assessed in terms of their effect on cell size (figure 6.1) and their capacity to adhere to plastic culture surfaces (figure 6.2). Culture supernatants were either diluted by the addition of PBS or concentrated by centrifugation to give a range of protein concentrations from 0.2mg/ml to 2mg/ml.

The longest axis of 20 macrophages were measured following 24 hour incubation in suspension with culture supernatants and subsequent smearing onto glass slides. The mean cell length of untreated cells was 6.7μm. Treatment with LCS supernatants at all protein concentrations had no significant effect on cell size. Similarly, treatment with MC and LC supernatants did not affect cell size.

Figure 6.2 shows the effect of culture supernatants on macrophage adherence to culture surfaces which in untreated samples was approximately $1x10^6$/ml from the addition of $1x10^7$/ml of viable cells. When incubated with LCS supernatants at 2mg/ml and 1mg/ml protein the number of adherent cells fell significantly to approximately $0.5x10^6$/ml and $0.6x10^6$/ml respectively. Treatment of macrophages with LCS at lower protein concentrations did not cause significant reduction in cell adherence.

This same pattern of reduced adherence at higher protein concentrations is repeated for MC supernatant treated cells. Incubation with MC supernatants at 0.5mg/ml and 0.4mg/ml however had no significant effect on macrophage adherence. By comparison, the reduction in cell adherence caused by LC supernatants is much less significant than that described above. However, these supernatants did cause significant reductions in adherence at 0.5mg/ml and 0.2mg/ml to $0.85x10^6$/ml and $0.8x10^6$/ml respectively. Treatment with LC supernatants at 0.4mg/ml protein did not significantly affect cell adherence properties.

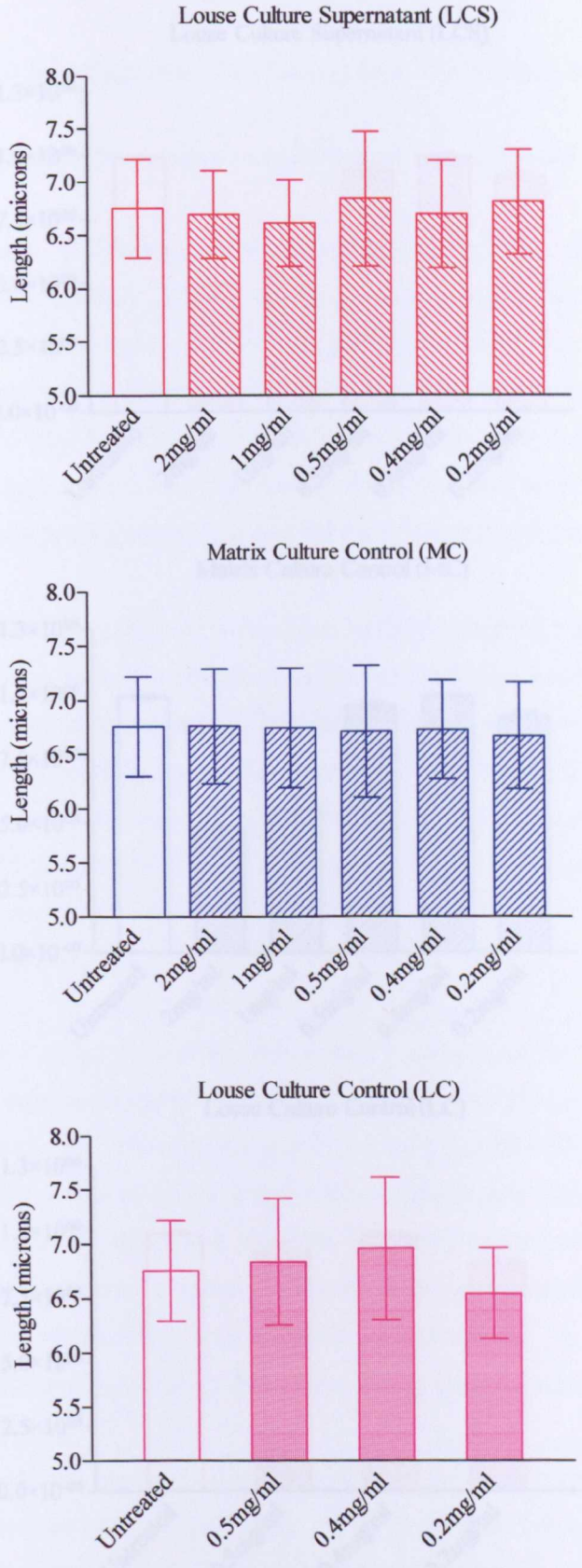


Figure 6.1

The effect of incubating salmon macrophages with culture supernatants at a range of protein concentrations on cell size. Treatments were applied to cells for 24 hours and data represent mean length (± standard deviation) of 20 macrophages per treatment.

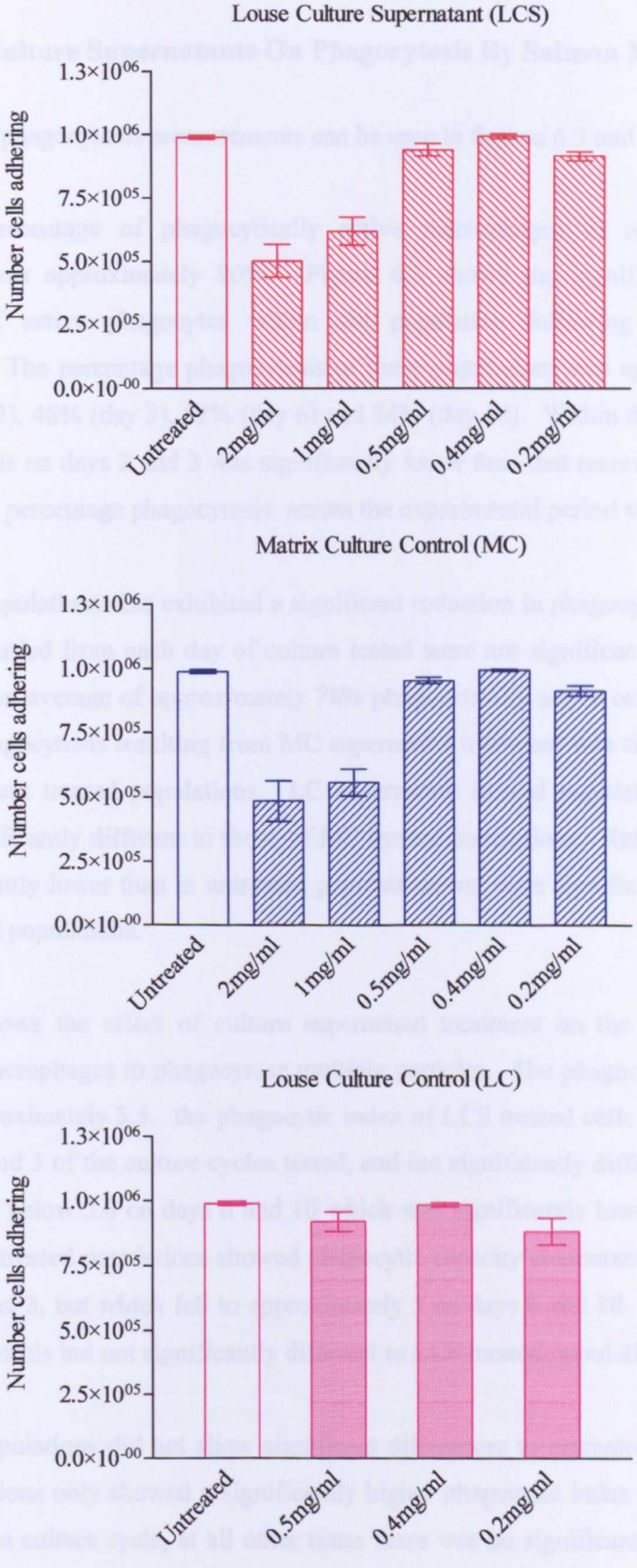


Figure 6.2

The effect on the adherence properties of salmon macrophages following 24 hour incubation with culture supernatants at a range of protein concentrations. Data are mean number of adherent cells (± standard deviation) calculated from duplicate culture wells per treatment.

Effects Of Culture Supernatants On Phagocytosis By Salmon Macrophages

The results of phagocytosis measurements can be seen in figures 6.3 and 6.4.

The mean percentage of phagocytically active macrophages in non-supernatant treated populations was approximately 90%. Figure 6.3 shows the significant reduction in the proportion of active phagocytes within the population following treatment with LCS supernatants. The percentage phagocytosis of these populations was approximately 50% (day 1), 46% (day 2), 46% (day 3), 52% (day 6) and 54% (day 10). Within these treatments the rate of phagocytosis on days 2 and 3 was significantly lower than that recorded on both days 6 and 10. The mean percentage phagocytosis across the experimental period was 49.6.

MC treated populations also exhibited a significant reduction in phagocytosis at all time points. The rates recorded from each day of culture tested were not significantly different from each other, giving an average of approximately 78% phagocytically active cells. At each time point the rate of phagocytosis resulting from MC supernatant treatment was significantly higher than LCS supernatant treated populations. LC supernatant treated populations had phagocytosis rates not significantly different to those of MC treated populations. Rates in these populations were significantly lower than in untreated populations but were significantly higher than those in LCS treated populations.

Figure 6.4 shows the effect of culture supernatant treatment on the phagocytic index; the capacity of macrophages to phagocytose multiple particles. The phagocytic index of untreated cells was approximately 3.5. the phagocytic index of LCS treated cells was approximately 3.2 on days 1, 2 and 3 of the culture cycles tested, and not significantly different to untreated cells. The index fell below 3.0 on days 6 and 10 which was significantly lower than untreated cells. Similarly MC treated populations showed phagocytic capacity comparable to untreated controls on days 1,2 and 3, but which fell to approximately 3 on days 6 and 10. This was significantly lower than controls but not significantly different to LCS treated populations.

LC treated populations did not show significant differences to untreated controls at any time. These populations only showed a significantly higher phagocytic index than LCS treated cells on day 6 of the culture cycle, at all other times there was no significant different between any culture supernatant treated population.

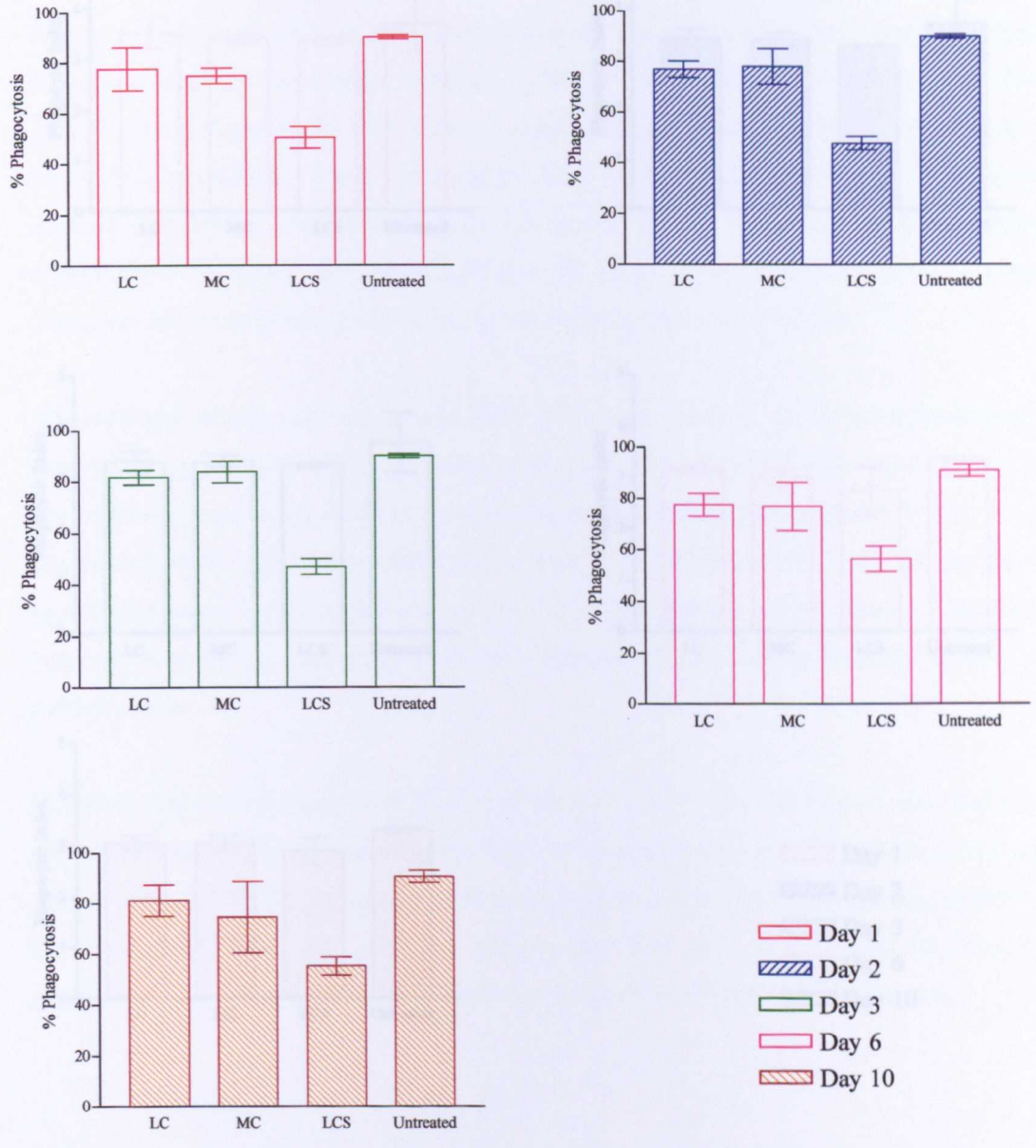


Figure 6.3

The effect on the ability of salmon macrophages to phagocytose a suspension of yeast following treatment of cells with culture supernatants. Cells were incubated with supernatants collected at 5 time points during culture cycles 2,3 and 4. Data are mean percentage of the macrophage population (± standard deviation) undergoing phagocytosis. In this experiment cells from 16 fish were used and 200 cells per treatment observed.

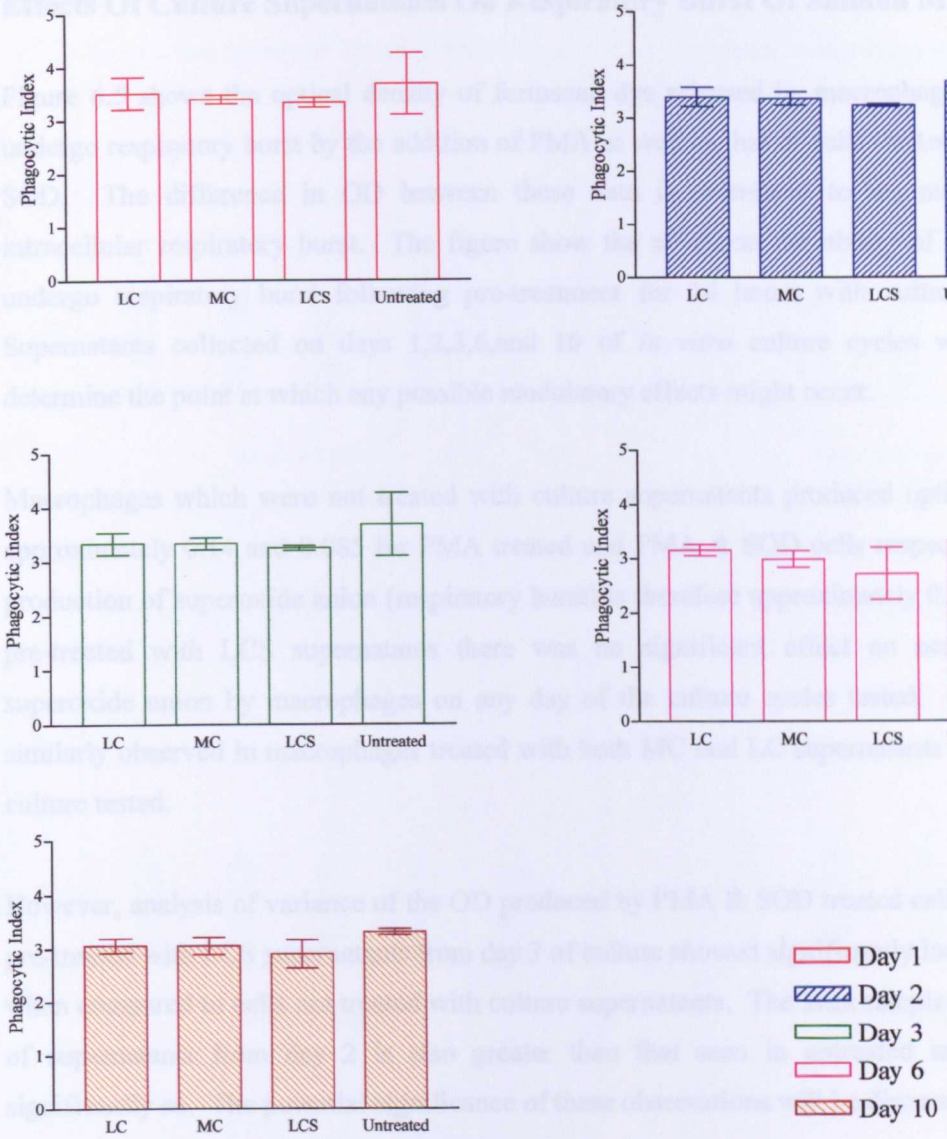


Figure 6.4

The effect on the capacity of salmon macrophages to phagocytose a suspension of yeast following treatment of cells with culture supernatants. Macrophages were incubated with supernatants collected at 5 time points during culture cycles 2,3 and 4. Data are mean number of yeast (± standard deviation) phagocytosed by individual macrophages. In this experiment cells from 16 salmon were used and 200 cells per treatment observed.

Effects Of Culture Supernatants On Respiratory Burst Of Salmon Macrophages

Figure 6.5 shows the optical density of formazan dye released by macrophages stimulated to undergo respiratory burst by the addition of PMA as well as that of cells treated with PMA and SOD. The difference in OD between these data is correlated to the magnitude of the intracellular respiratory burst. The figure show the effect on the ability of macrophages to undergo respiratory burst following pre-treatment for 24 hours with culture supernatants. Supernatants collected on days 1,2,3,6,and 10 of *in vitro* culture cycles were assayed to determine the point at which any possible modulatory effects might occur.

Macrophages which were not treated with culture supernatants produced optical densities of approximately 0.14 and 0.085 for PMA treated and PMA & SOD cells respectively. The net production of superoxide anion (respiratory burst) is therefore approximately 0.055 OD. When pre-treated with LCS supernatants there was no significant effect on net production of superoxide anion by macrophages on any day of the culture cycles tested. This result was similarly observed in macrophages treated with both MC and LC supernatants on all 5 days of culture tested.

However, analysis of variance of the OD produced by PMA & SOD treated cells that were also pre-treated with LCS supernatants from day 3 of culture showed significantly increased variance when compared to cells not treated with culture supernatants. The intra sample variation in OD of supernatants from day 2 is also greater than that seen in untreated samples, but not significantly so. The potential significance of these observations will be discussed.

Effects Of Culture Supernatants On Chemotaxis Of Salmon Macrophages

Figure 6.6 shows the effects in vitro culture supernatants had on the ability of salmon macrophages to migrate towards a known chemoattractant.

Approximately 150 cells were observed, per field of view, passing through a 5μm membrane separating the upper and lower (attractant containing) compartments of a chemotaxis chamber. The pre-treatment of macrophages with LCS supernatants collected on day 1 of the culture cycle resulted in a greatly significant reduction in the number of migrating cells to approximately 65. The incubation of cells with LCS supernatants collected on days 2,3,6 and 10 of culture also

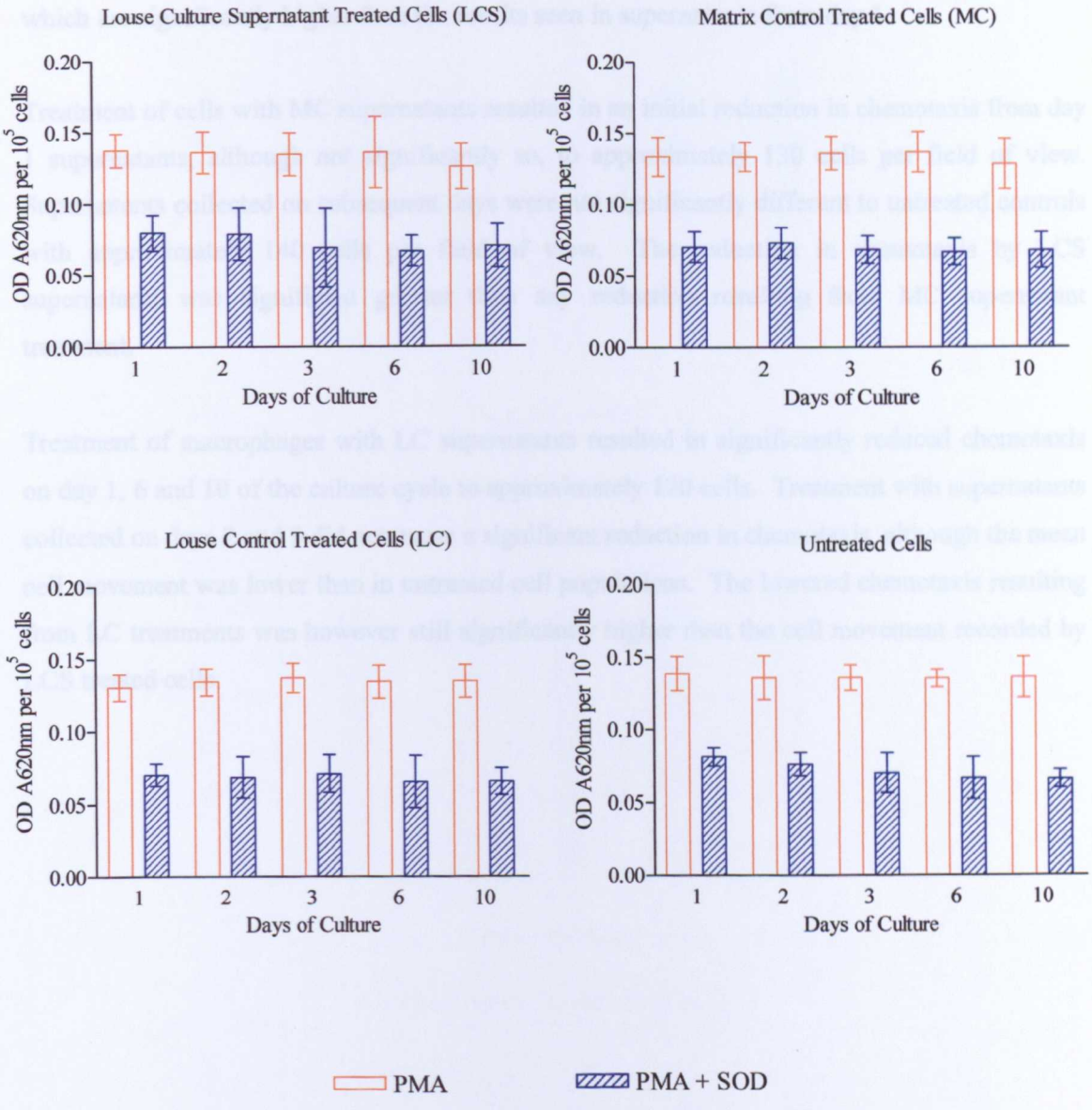


Figure 6.5

The effect of pre-treatment of salmon macrophages with culture supernatants on their ability to undergo intracellular respiratory burst. Data represent mean optical density (OD) of formazan dye released by both PMA stimulated cells and cells treated with PMA and SOD. The difference in OD is correlated to the magnitude of the respiratory burst. In these experiments macrophages from 16 fish were incubated with supernatants collected at 5 time points during culture cycles 2, 3 and 4.

resulted in significantly reduced chemotaxis to between 75 and 100 cells per field of view, which are significantly higher than the results seen in supernatants from day 1.

Treatment of cells with MC supernatants resulted in an initial reduction in chemotaxis from day 1 supernatants, although not significantly so, to approximately 130 cells per field of view. Supernatants collected on subsequent days were not significantly different to untreated controls with approximately 140 cells per field of view. The reduction in chemotaxis by LCS supernatants was significant greater than any reduction resulting from MC supernatant treatment.

Treatment of macrophages with LC supernatants resulted in significantly reduced chemotaxis on day 1, 6 and 10 of the culture cycle to approximately 120 cells. Treatment with supernatants collected on days 2 and 3 did not cause a significant reduction in chemotaxis, although the mean cell movement was lower than in untreated cell populations. The lowered chemotaxis resulting from LC treatments was however still significantly higher than the cell movement recorded by LCS treated cells.

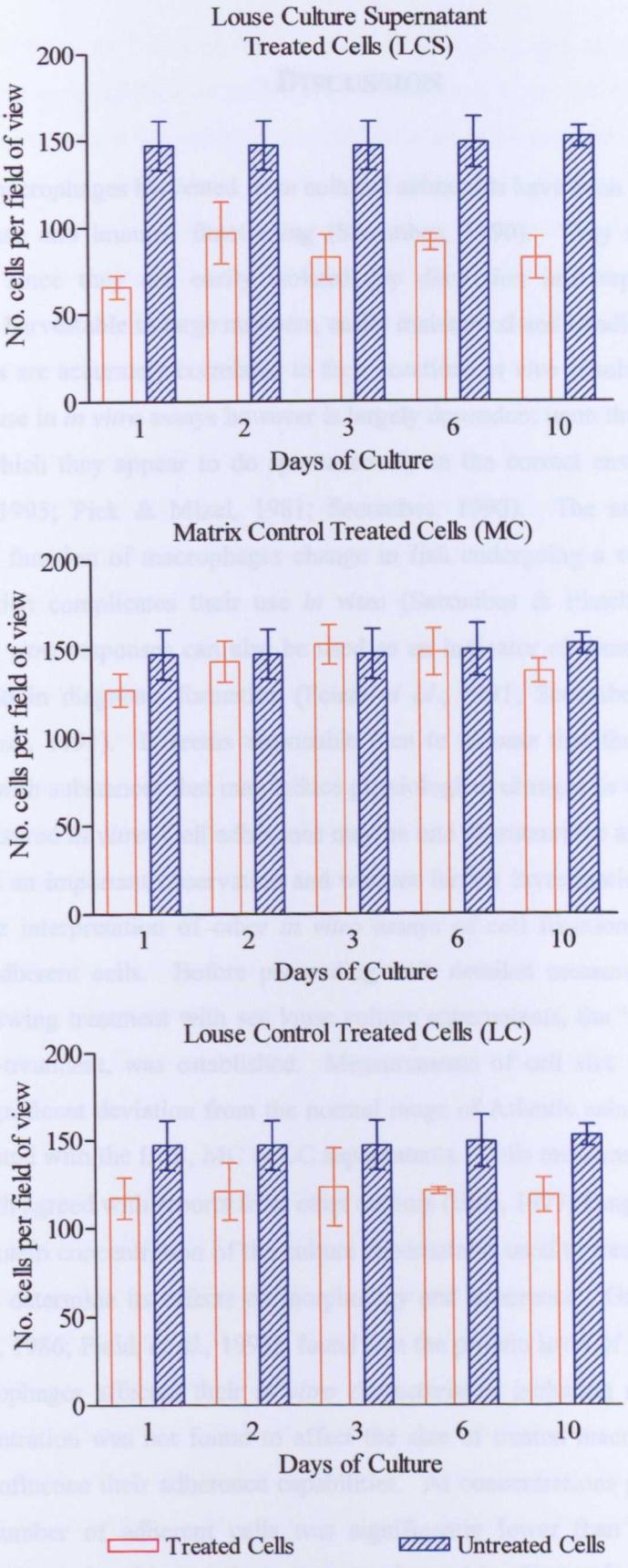


Figure 6.6

The effect on the chemotaxic ability of salmon macrophages following treatment with culture supernatants. Cells from 16 Atlantic salmon were incubated with supernatants collected from 5 time points during culture cycles 2,3 and 4. Data are net movement of cells towards a chemoattractant and represent the mean (± standard deviation) of 12 chemotaxis chambers observed per treatment.

DISCUSSION

Head kidney macrophages harvested from cultured salmonids have been used extensively in the study of disease and immune functioning (Secombes, 1990). They represent a significant research tool since they are easily isolated (by dissection and separation using density gradients), are harvestable in large numbers, easily maintained and handled *in vitro*, and their *in vitro* responses are accurately correlated to their reactions *in vivo* (Paulnock, 2000; Secombes, 1990). Their use in *in vitro* assays however is largely dependent upon their adherence to plastic cultureware which they appear to do spontaneously in the correct environment (Ellis, 1977; Field *et al.*, 1995; Pick & Mizel, 1981; Secombes, 1990). The adherence, morphology, chemistry and function of macrophages change in fish undergoing a variety of physiological responses, which complicates their use *in vitro* (Secombes & Fletcher, 1992). However, unexpected *in vitro* responses can also be used as an indicator of these wider changes in the animal and aid in diagnosis formation (Peters *et al.*, 1991; Secombes and Fletcher, 1992; Waterstrat *et al.*, 1991). It seems reasonable then to assume that the treatment of isolated macrophages with substances that may induce physiological changes *in vitro,* might affect their responses measured *in vitro*. Cell adherence may be one characteristic affected, and whilst that could stand as an important observation and warrant further investigation in itself, it will also complicate the interpretation of other *in vitro* assays of cell functioning which rely on the presence of adherent cells. Before proceeding with detailed measurements of macrophage functions following treatment with sea louse culture supernatants, the 'normality' of the cells, pre- and post-treatment, was established. Measurements of cell size were conducted which showed no significant deviation from the normal range of Atlantic salmon macrophages when cells were treated with the LCS, MC or LC supernatants. Cells measured were in the range 6.0 to 7.5μm which agreed with reports from other authors (Ellis, 1977; Fange, 1994; Rowley *et al.*, 1988). The protein concentration of the culture supernatants used to treat the macrophages was also varied to determine its effects on morphology and adherence. Graham (1988), amongst others (Bayne, 1986; Field, *et al.*, 1995), found that the protein level of media used to maintain isolated macrophages affected their *in vitro* characteristics including adherence. Whilst the protein concentration was not found to affect the size of treated macrophages in the current study, it did influence their adherence capabilities. At concentrations greater than 1mg/ml of protein the number of adherent cells was significantly lower than in untreated controls. However, at 0.5mg/ml and below there were no observable effects of any of the supernatants. The previously cited studies noted effects due to the protein concentration of media containing Foetal Calf Serum (FCS), where the exact nature and reasons for these effects were unclear

(Field, *et al.*, 1995). FCS is a mixture of various biological and immunological factors, the combination and concentration of which may be responsible for the changes caused. ASSE, early in its construction, is supported in media containing FCS, and even though it is removed in later stages it may still exert some influence by leaching from lower layers through damage caused to the substrate, and forming part of the culture supernatant. However, there were no observed differences in the influence of LCS protein concentration and the protein level of MC supernatants, which would suggest that the changes in adherence were not influenced by the presence of copepodid products.

The reductions in adherent cells following treatment with LCS and MC supernatants were not significantly different to each other. This suggests that the effects were not influenced by the presence of copepodid products in the supernatants, and might therefore have been caused by residual serum in the ASSE.

Having determined that macrophages treated with culture supernatants were normal, in terms of their morphology and adherence characteristics, the effects on the cells' immunological functions were measured. Broadly speaking, Matrix control (MC) and Louse control (LC) supernatants did not significantly modify the functions or effectiveness of the cellular immune parameters investigated. Louse Culture supernatants (LCS) however, did result in significant negative effects on the phagocytic activity and chemotaxis of the macrophage populations, but did not affect the cells' phagocytic index or respiratory burst capabilities. These effects were evident in supernatants collected from the first day of culture and persisted in supernatants collected until day 10. The persistence of effects of supernatants collected beyond day 10 was not measured.

The lack of influence of MC and LC supernatants on the *in vitro* performance of salmon macrophages indicates firstly that the ASSE is immunologically inert (with regard to these functional assays) and secondly, that any effects attributed to *L. salmonis* copepodids occur only when the larvae are settled on the ASSE substrate and not when they are free-swimming. Arthropod parasites, particularly haematophagus parasites, employ an array of bioactive molecules with potent effects on the hosts' immune system, both humoral and cellular (Wikel *et al.*, 1996). The most studied of these parasite groups, the ixoid ticks, secrete immunomodulatory compounds from salivary glands. Research into these species has advanced beyond measurement of the effects that salivary extracts have on gross immune functions and instead is focused on the biochemical processing of metabolic information that cause the effects on antibody and cell-mediated defences. This work is now beginning to identify the specific

pathways and feed back mechanisms that parasite secreted products affect and which have broader effects that include reduction in macrophage activity, chemotaxis, phagocytosis, respiratory burst and cytokine production (Wikel & Osbourne, 1982;Wikel, 1985; Wikel, 1988; Ramachandra & Wikel, 1992; Wikel *et al.*, 1994; Wikel, *et al.*, 1996). The data provided within this thesis presents the first demonstration of the immunomodulatory products produced by *L. salmonis*. Others have suggested the presence of such compounds but have either failed to demonstrate this experimentally (Johnson & Albright, 1992), or have suggested alternative explanations, such as the stress caused by physical injury during louse infection, after performing immunological analyses of infected salmon (Mustafa *et al.*, 2000). The current evidence, whilst eloquently describing the suppression of aspects of salmon cellular immunity, does not provide sufficient evidence to elaborate on the nature and mode of action of sea louse secreted/excreted active products, which are tentatively designated Louse Immunomodulatory Factor (LIF). However, the evidence of LIF effects on salmon macrophages, and supporting evidence of immuno-modifying compounds used by other parasites does permit a degree of speculation of what is after all a preliminary investigation into the production of such compounds by this particular parasite.

LCS treated macrophages (i.e. those exposed to LIF) have a significantly reduced phagocytic activity, approximately 50% of the cell population was inactive, compared to only about 10% of the untreated population. However, those LIF treated cells that are phagocytically active were not hindered in terms of their capacity to phagocytose, and had a phagocytic index comparable to that of untreated cells. Several possibilities present themselves:

I. The secreted product(s) (LIF) in some way influence the motility of treated cells, since phagocytosis involves elements of chemotaxis and the directional movement of pseudopodia along a chemical gradient towards foreign material, and physical stimulation of the cell membrane (Paulnock, 2000). LIF exhibits significant inhibitory effects on macrophages during chemotaxis assays, which may also influence the chemotaxis elements of phagocytosis assays. Those cells in close association with yeast during phagocytosis assays are stimulated to engulf them through physical contact, whilst those located distally from large aggregations of yeast are not able to move to phagocytose the cells and are recorded as being phagocytically inactive.

II. Macrophage populations are composed of sub-populations of cells that are discriminated by both their maturity and their state of activation (Secombes and Fletcher, 1992) which may influence their susceptibility to the effects of LIF.

Similarly, these same factors affect the production of cytokines, such as macrophage activating factor (MAF), *in vitro* which is dependent upon the activation level of cells which often require exogenous stimulation to produce measurable quantities of the compound (Chung & Secombes, 1987; Secombes, 1990). Cells within a population may be more or less susceptible to the effects of LIF if they are mature and in an active state, or *vice versa.*

The lack of effect of LIF on macrophage respiratory burst capabilities suggests two possibilities; 1) that it does not directly interfere with oxidative defence pathways of macrophages, or 2) that it does not interfere with macrophage activity or immune functioning at all. This would seem contradictory considering the recorded effects on phagocytosis and chemotaxis, however, it can be better explained by considering all of the effects on cellular immunity highlighted by the experiments in this chapter as a single effect. LIF does not appear to affect a cells' ability to phagocytose or to go through respiratory burst, but does significantly reduce chemotaxis. As has already been mentioned, the reduction in phagocytic activity of LIF treated cells can be explained as an effect on chemotaxis rather than on the actual process of particle engulfment. Chemotaxis requires the reception of a chemical signal by the cell to instigate its movement along the chemical gradient produced by the signalling compound. If the cell is prevented from either receiving the signal, or misinterprets the signal, then the desired outcome (chemotaxis) will not occur, even though the cell may be perfectly capable of performing its other functions normally (Paulnock, 2000). Since phagocytosis and respiratory burst functions are still operational in LIF treated cells it could be suggested that its immunomodulatory function is directed towards the chemotaxic mechanisms of macrophages.

As previously mentioned, chemotaxis involves the reception of a stimulus, its interpretation and then action by the cell. If the hypothesis is to be accepted that LIFs primary effect is directed towards cell chemotaxis, it then remains to determine the stage of the chemotaxis process that it appears to target. It may be that LIF acts as a pharmacological antagonist that binds to receptors that provide directional information and that normally initiate changes in cell physiology that permit movement. Blocking of these receptors does not initiate the required changes and the cell is not stimulated to move. Alternatively, LIF may affect cell motility. In this instance, the signal is received by the cell and interpreted as a stimulus to move but the cell is physically incapable of doing so. The process of cell movement involves complex cytoskeletal changes but is ultimately regulated by the permeability of calcium channels in the cell membrane, and the movement of calcium ions into the cell (Lee *et al.*, 1999). After the movement 'event', the calcium channels close and the movement of calcium inside the cell

falls. Drugs, such as Nifedipine, typically used in the treatment of angina, function as calcium antagonists that block entry of calcium into cells to regulate movement of cardiac muscle cells (Rang *et al.*, 1995), however, evidence of calcium antagonist compounds secreted by parasitic organisms is not known even given the wealth of investigation into the mechanisms of parasitic immunomodulation.

Lastly, LIF may function by interfering in the cells' interpretation of the chemotaxic stimulus, and prevent the reception of exogenous chemical signals from initiating complex pathways within the cell that produce the desired outcome. The blocking or disruption of any part of these pathways could mislead the macrophage into producing an inappropriate response, or no response at all. The involvement of parasite secreted compounds that operate in this manner, primarily by the modulation of the intracellular messenger cAMP, have been implicated in the immunomodulation, and strategies for successful parasitism, employed by *Trypanosoma cruzi* and *Leishmania donovani*. In the case of *T. cruzi*, a serine peptidase, oligopeptidase B (OPB), is secreted that mobilises calcium ions from intracellular stores within the host and prevents their incorporation in pathways regulating invasion by fibroblasts, epithelial cells and myoblasts. Deletion of the OPB gene from parasites results in significant reductions in intracellular calcium ion concentrations when compared to the 'wild-type' parasite, and a slightly reduced invasion capacity. However, *T cruzi* also stimulates elevation of host cAMP as a mechanism of interference in intracellular pathways which OPB-null parasites were still able employ to allow parasitism. This demonstrates effectively both the possibility of parasites manipulating intracellular communications to enhance their survival, and also the complex mechanisms and use of multiple compounds that they have evolved to disrupt normal functioning. *L. donovani* promastigotes are also known to employ methods of interfering in intracellular communication pathways. The precise mechanism is not fully understood, however, some involvement in cAMP manipulation is thought likely. Stimulation of parasite infected cells with exogenous cAMP and compounds known to stimulate cAMP production in macrophages, results in elevated levels of cAMP and inhibition of superoxide dismutase (SOD) which is a key enzyme in the regulation of the oxidative defence system. As well as dismutasing superoxide to hydrogen peroxide, a more potent reactive oxygen species, SOD is also the first step in the down regulation of superoxide and in sufficient concentrations can actually prevent respiratory burst. By modulating the level of cAMP in the macrophage, the parasite prevents the inhibition of SOD which in turn prevents the cell from mounting an effective oxidative defence against the parasite.

However, there exists no direct evidence from the experiments conducted in the present study to determine either the mode of action or the biochemical nature of LIF, and all of the hypotheses suggested above are purely speculative at this stage. A better understanding of the other immunological and physical effects of LIF would permit the streamlining of future biochemical investigation. For instance, nothing is known about the permanency of the effects of LIF and whether if following a period of incubation in non-LIF containing media, the immunological effects remain. Also, it would be beneficial to understand if LIF treated macrophages are still able to produce cytokines such as MAF and TNFα. A lack of ability may suggest the modulation of the cells' metabolism of inflammatory mediators and its ability to communicate intercellularly.

Ribeiro (1995) describes ticks as 'smart pharmacologists' since they employ a sophisticated arsenal of immunomodulatory countermeasures to host defences based on metabolic compounds operating within the host which they use to close or reverse pathways that would otherwise prevent their parasitism. Amongst these compounds are substances involved in cell signalling and communication both intra- and inter-cellularly. Eicosanoids, primarily prostaglandins, and specifically prostaglandin E_2 (PGE_2), are active components of tick salivary gland extracts (Wikel, *et al.*, 1994; Wikel, *et al.*, 1996). However, prostacyclin, a related product of cycloxygenase metabolism is present in the saliva of *Ixodes dammini* (Ribeiro *et al.*, 1985) and has comparable effects to PGE_2 in this species. Research has attributed a vast number of effects of tick salivary gland extracts to PGE_2, to the extent that it is broadly accepted as a primary method of 'general' immunosuppression used by this group of parasites (Inokuma *et al.*, 1994; Urioste *et al.*, 1994; Wikel, *et al.*, 1994; Wikel, *et al.*, 1996). However, additional substances have significant immunomodulatory effects, the majority of which still remain largely unclassified (Wikel, *et al.*, 1994; Wikel, *et al.*, 1996). However, a 49kDa protein from *I. dammini* has been identified that has anticomplement activity (Ribeiro, 1987), as has a salivary carboxypeptidase B that functions as an anaphylatoxin inhibitor (Ribeiro & Spielman, 1986). Additionally, a 5 kDa protein has been identified that exhibits inhibition of murine lymphocyte blastogenesis (Urioste, *et al.*, 1994) as well as a 65kDa protein that functions as an anticoagulant (Limo *et al.*, 1991). Therefore, whilst the biochemical nature of LIF may have a single, dominant, easily identifiable component, as PGE_2 is in ticks (although this is not a suggestion that the same compound operates in LIF), it may also consist of others that are less readily classified, and whose effects are complex, possibly masked by those of the others, but which are nevertheless potent immunomodulators.

It was outside the scope of this thesis to identify the sea louse immunomodulatory compounds and their mode of action. Prior to the start of this work, the presence of such compounds had only been theorised as an explanation for the apparent lack of immune response of Atlantic salmon to the parasite, with no experimental evidence to prove its existence. The intention was simply to determine whether *L. salmonis*, cultured *in vitro*, produced any compounds which could modulate the functions of immune cells *in vitro*, and for these products to be collected and assayed for their specific immunomodulatory effects. The project has succeeded in this and has opened many avenues for further investigation. Extensive biochemical investigation is not yet possible until the full effect of LIF on macrophage functioning can be established. Success in this should narrow the field of potential candidate compounds and permit the required study. However, the physical properties and preliminary biochemical characteristics of the active components of LIF are more easily identifiable and are investigated in the next chapter of this thesis.

PRELIMINARY BIOCHEMICAL PROPERTIES OF CULTURE SUPERNATANTS

Chapter 7

INTRODUCTION

THE PRELIMINARY BIOCHEMICAL PROPERTIES OF CULTURE SUPERNATANTS

The development of techniques and methods for protein purification and identification has been an essential pre-requisite for many of the advancements made in biotechnology. Protein purification varies from simple one-step precipitation or colourmetric procedures to large scale production processes often involving multiple purification steps to reach the desired purity. The choice of technique is dependent upon the scale of the operation, the purity required, the stage of the analytical process, the nature and the volume of the initial sample for processing. Typically most purification and accurate identification schemes involve some form of chromatography which offers many varied techniques and combinations of methodologies to allow the analysis of virtually any biomolecule (Amersham Pharmacia Biotech, 1998).

Prior to the use of technically complex analytical procedures such as chromatography, the identity of biological substances can be characterised using a range of more simple techniques. These will allow the determination of the biochemical nature of the compound; whether it is protein, lipid carbohydrate or nucleic acid, as well as defining some of the parameters within which it is biologically active such as its optimum pH and temperature (Miller, 1990). Typically, enzyme assays, and commercially available kits (API ZYM, Marcy l'Étoile, France) that use enzyme specific substrates to identify the enzymatic profile of test material are the starting point in research, as well as forming the basis of preliminary diagnostic tests in many laboratories. Such techniques have been used to classify the activity of and identify the active protein components of specimens in all fields of biology (Price & Stevens, 1999). Simple biochemical analysis of material displaying enzymatic properties can be used to determine the enzyme class to which is belongs. These tests can, for example, identify whether the enzymatic reactions involve the addition or removal of water (hydrolase or hydrase enzymes), the transfer of electrons (oxidases or dehydrogenases) or the change in geometry or structure of a molecule (isomerases) (Miller, 1990). Testing can also determine the activity at varying substrate and enzyme concentrations, pH, and temperature which will enable more specific classification of the protein and will allow identification based on the same reactions documented in reference sources such as the Worthington Enzyme Manual (1988). More specific assays test the proteins' reaction towards known enzyme specific substrates, which are also available in commercially prepared kit form. The API ZYM system is a colourmetric test kit that assays for

the activity of 19 common enzymes in a semi-quantitative way. It is used extensively in the identification of bacteria, bacterial products and biological contaminants of food products (Samolada *et al.*, 1998), in public health (Bridge & Hawsworth, 1984; Ling *et al.*, 1994), marine ecology (Roberts *et al.*, 1997) and in human and veterinary medical diagnostics and research (Levett, 1985; Nisbet & Billingsley, 1999). This system has been used in the study of *L. salmonis* by Roberts *et al.* (1999). In this work the authors used API ZYM tests to identify the enzymatically active components of the louse gut to determine the physiological environment in which host antibodies might need to survive in order that vaccines based on targeting louse gut antigens might be successful. They identified the presence, in high concentrations, of the enzymes leucine aminopeptidase, valine aminopeptidase, N-acetyl-glucosaminidase, alkaline phosphatase and acid phosphatase but were at a stage of research where speculation of their significance was not possible.

Chromatographic methods allow accurate quantitative measurements of the biochemical constituents of substances that are far more advanced than the techniques previously described. The identity of unknown compounds can be determined in several ways using chromatographic systems including ion exchange, gel filtration and affinity chromatography, amongst others (Amersham Pharmacia Biotech, 1998). Chromatography of these kinds have been used in many aspects of fish research including the identification of fish species based on their chromatographic profiles (Knuutinen & Harjula, 1998), the discrimination of wild and farmed salmon escapees in river systems (Poole *et al.*, 2000), the identification of chemical residues in fish tissue (Meinertz *et al.*, 1998), the isolation and identification of fish metabolites (Fukada *et al.*, 1996; Moriyama *et al.*, 1997), and the analysis of immunogenic (Stensvag *et al.*, 1999) and immunosuppressive components (Fredriksen *et al.*, 1997) of organisms pathogenic to farmed fish species. These techniques have also been applied to the study of *L. salmonis* and their infection of farmed salmonids, both in the identification of potential vaccine antigens (Grayson *et al.*, 1995; Roper *et al.*, 1995), and the analysis of salmon integumental biochemistry during sea lice infection (Firth *et al.*, 2000). In the latter research the authors used protein and enzymological techniques to identify a series of low molecule weight (17-22kDa) serine proteases in the mucus of lice infected Atlantic salmon. These proteases were subsequently characterised as being trypsin-like, and associated with louse feeding mechanisms and not involved in host defence strategies.

This study will use gel filtration chromatography to investigate the protein profile of *L. salmonis* culture supernatants. This technique is a research and diagnostic tool used in all fields of biological, geological and materials sciences which separates proteins with differences in

molecular size in a single buffer that allows the proteins to be collected in a purified form (Robards *et al.*, 1994) which will permit the collected culture supernatant fractions to be further investigated.

MATERIALS AND METHODS

This chapter describes standard biochemical methodologies and techniques used to identify the nature of the biomolecules present within louse culture supernatants that demonstrated chemotaxic suppression of salmon macrophages assayed in chapter 6. Preliminary analysis measured the total protein content of the supernatants (section 2.33) and then examined the effect on immunosuppressive activity of heating and diluting of the supernatants (section 2.34) by measuring their effects on macrophage chemotaxis following treatment (using the method described in section 2.11). Confirmation of the proteinaceous nature of the immunosuppressive components was assayed by the incubation of supernatants with the broad-spectrum endopeptidase Proteinase K (section 2.35). Culture supernatants were also analysed by the API ZYM system to produce enzyme activity profiles, which could be used to detect differences between each supernatant group and between culture cycles (section 2.36). Gel filtration chromatography was used to visualise, collect and characterise the size of proteins and polypeptides within each culture supernatant (section 2.37). Analytical software used in this technique permitted isolation of proteins that were present only in the Louse Culture Supernatant (LCS) which were subsequently tested for immunosuppressive activity towards salmon macrophage chemotaxis (section 2.38).

RESULTS

THE PRELIMINARY BIOCHEMICAL PROPERTIES OF CULTURE SUPERNATANTS

The Protein Content Of Culture Supernatants

Figure 7.1 shows the changes in protein concentration of culture supernatants during the progression of the culture cycle. The LCS supernatants show an increasing concentration as the cycle continued. Following 24 hours of copepodid incubation the protein level was approximately 0.5mg/ml. This content appeared to plateau until rising again at day 6, with the trend continuing until the level peaked at approximately 1.3mg/ml by day 12 of culture.

MC supernatants contained approximately 0.3mg/ml protein at day 1 which was maintained until day 9 when the concentration increased to approximately 0.9mg/ml by the end of the experiment at day 14. The protein concentration of these supernatants was significantly lower than that of LCS supernatants at each day of culture tested, except at the start of the experiment when the maintenance of ASSE in 0.001M PBS prior to the addition of seawater produced a protein measurement of approximately 0.2mg/ml.

The protein concentration of LC supernatants remained low at approximately 0.15mg/ml but showed a gradual increase from day 6. The concentration peaked at approximately 0.4mg/ml at day 10. On days 10, 11 and 12 of culture LC supernatants possessed comparable levels of protein to MC supernatants, however, with these exceptions the protein levels were significantly lower than those measured in both LCS and MC supernatants.

There was no measurable protein content in the dialysed seawater controls from the culture cycles. These data described above represent the mean of measurements from duplicate culture cycles that showed no significant differences in the protein content of supernatants at each day of sampling.

Figure 7.1a shows the net protein concentration of LCS supernatants. The higher content with LCS supernatants remains relatively stable for the first 7 days of the culture cycle at around 0.05mg/ml to 0.15mg/ml, but begins to increase significantly in subsequent days where it peaks at approximately 0.35mg/ml higher than the measurements from the control supernatants.

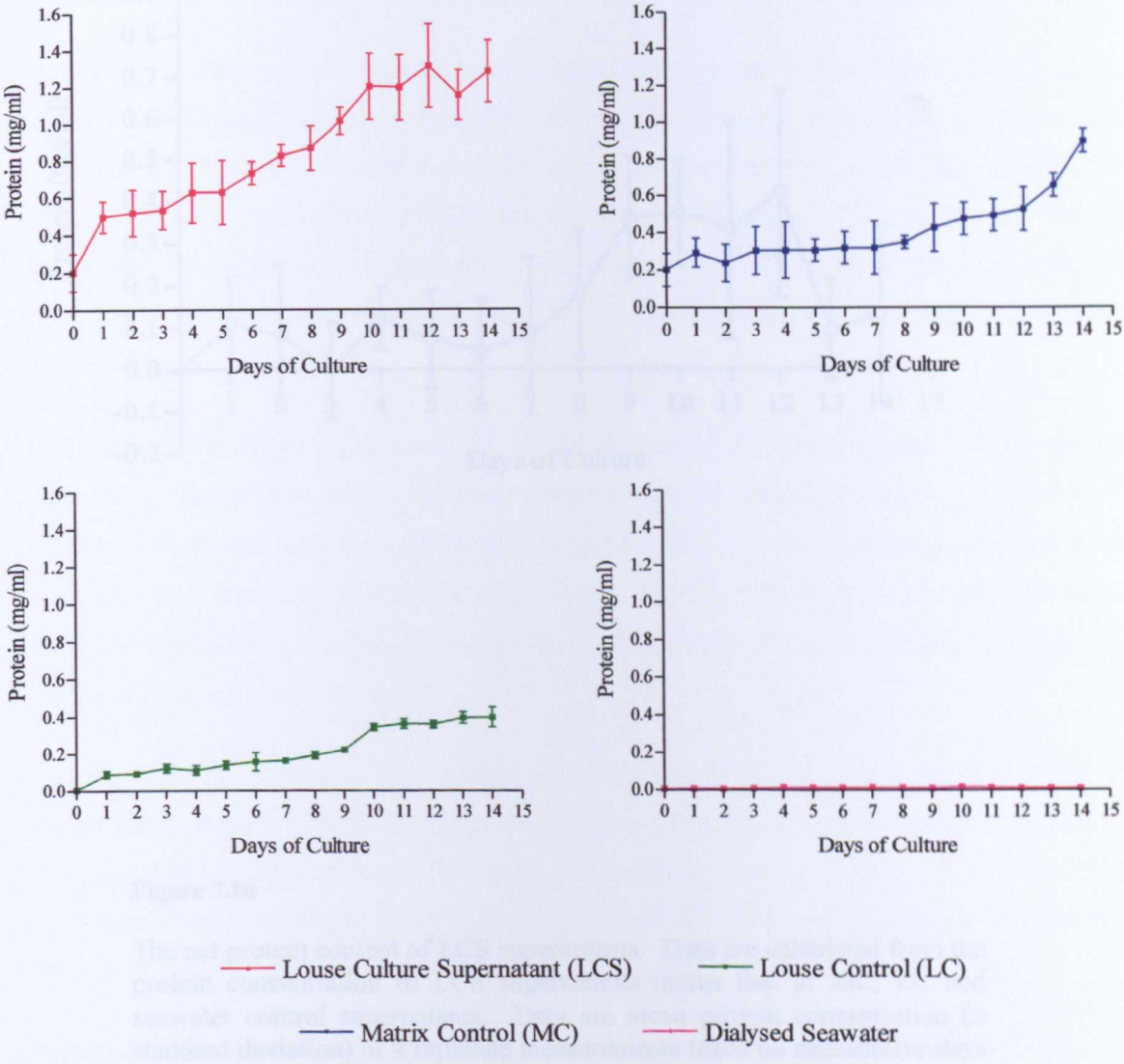


Figure 7.1

Measurements of the protein content of culture supernatants collected on consecutive days of 2 culture cycles. Data are mean protein concentration (± standard deviation) of 4 replicate measurements taken at each time point.

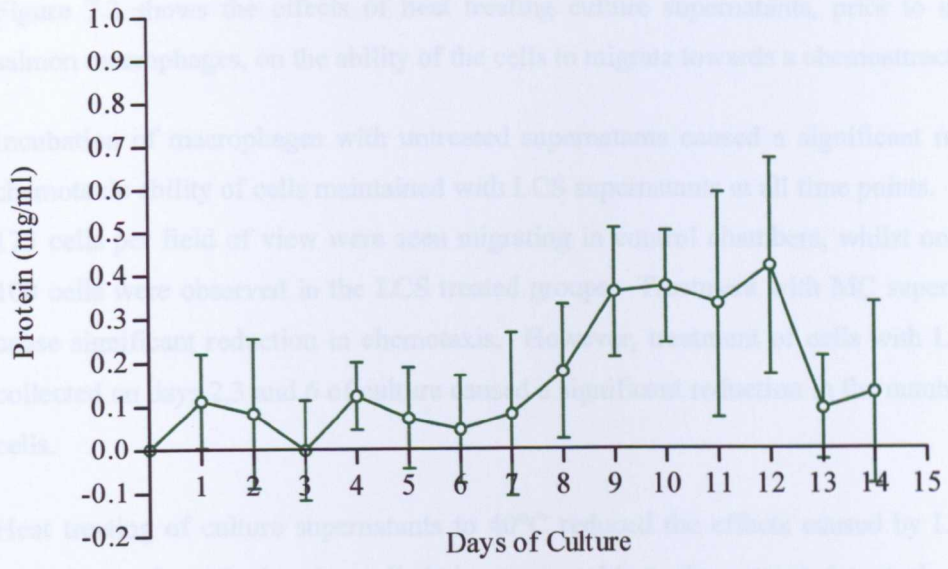


Figure 7.1a

The net protein content of LCS supernatants. Data are calculated from the protein concentration of LCS supernatants minus that of MC, LC and seawater control supernatants. Data are mean protein concentration (± standard deviation) of 4 replicate measurements taken on consecutive days of 2 culture cycles

The Effects Of Heat Treated Culture Supernatants On Chemotaxis of Salmon Macrophages

Figure 7.2 shows the effects of heat treating culture supernatants, prior to incubation with salmon macrophages, on the ability of the cells to migrate towards a chemoattractant.

Incubation of macrophages with untreated supernatants caused a significant reduction in the chemotaxic ability of cells maintained with LCS supernatants at all time points. Approximately 175 cells per field of view were seen migrating in control chambers, whilst only between 70-100 cells were observed in the LCS treated groups. Treatment with MC supernatants did not cause significant reduction in chemotaxis. However, treatment of cells with LC supernatants collected on days 2,3 and 6 of culture caused a significant reduction in the number of migrating cells.

Heat treating of culture supernatants to 40°C reduced the effects caused by LC supernatants with the number of migrating cells being comparable to the untreated controls. The ability of macrophages treated with MC supernatants heated to 40°C was not significantly reduced when compared with untreated controls and was not significantly different to results obtained when the same supernatants were not heat treated. In both these cases approximately 160 cells per field of view were observed migrating.

Heating LCS supernatants to 40°C caused no significant changes in the supernatants' ability to reduce macrophage chemotaxis. These supernatants still resulted in a significant reduction in cell migration to approximately 100 cells per field of view. There were no significant differences in the effects caused by these supernatants when collected at different times during the culture cycle.

Heating MC supernatants to 80°C still did not significantly change the number of migrating cells when compared to untreated control, or when compared to the same supernatants heated to 40°C or when non-heat treated. The number of cells migrating following treatment with LC supernatants heated to 80°C did not vary significantly from the number recorded when treated with LC supernatants heated to 40°C. In both cases the approximate number of migrating cells was 160 per field of view. Similarly, the rate of chemotaxis of these treated groups was not significantly different to that seen in untreated controls.

Treatment of macrophages with LCS supernatants heated to 80°C resulted in rates of chemotaxis that were significantly lower than seen in untreated controls, except for supernatants collected on day 10 of culture. However, the approximate rate of migration of these cells, 150 per field of view, was not significantly different to that seen in cells treated with either MC or

LC supernatants also heated to 80°C. Except for data recorded on day 6 of culture, the rate of chemotaxis measured from 80°C treated LCS supernatants was significantly higher than the same supernatants with lower or no heat treatment.

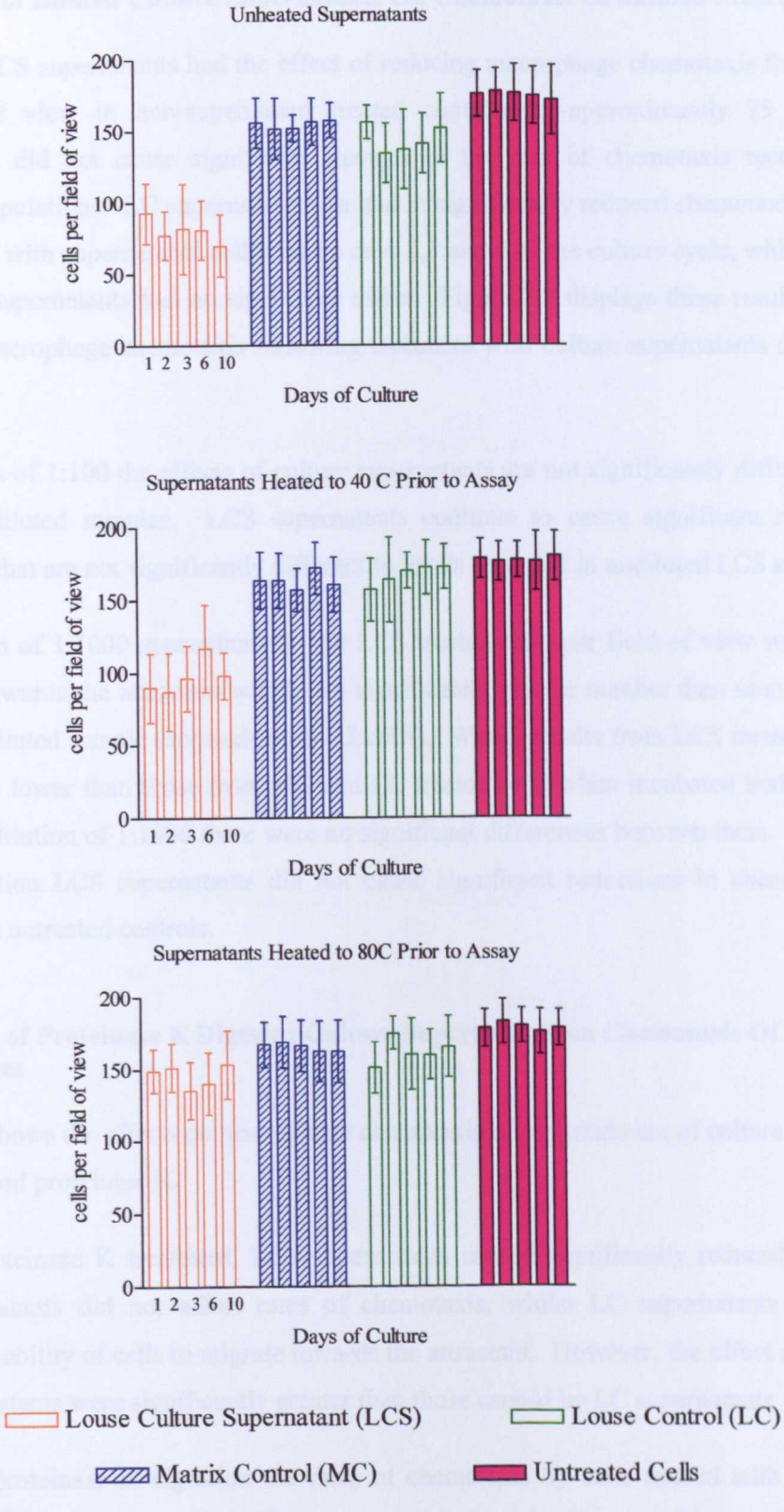


Figure 7.2

The effects of heat treated culture supernatants on chemotaxis by salmon macrophages. Data represent the net movement of cells towards an attractant and are the mean (± standard deviation) calculated from 12 chemotaxis chamber observations per treatment. Treatments are presented graphically as groups with each bar representing a single treatment pertaining to a supernatant collected from one day of a copepodid culture cycle (in the order day 1,2,3,6 & 10).

The Effects of Diluted Culture Supernatants On Chemotaxis Of Salmon Macrophages

Undiluted LCS supernatants had the effect of reducing macrophage chemotaxis from 175 cells per field of view in non-supernatant treated controls to approximately 75 cells. MC supernatants did not cause significant changes to the rate of chemotaxis recorded in the untreated populations. LC supernatants resulted in significantly reduced chemotaxis when cells were treated with supernatants collected on days 2,3 and 6 of the culture cycle, whilst day 1 and day 10 LC supernatants had no significant effect. Figure 7.3 displays these results as well as results of macrophage chemotaxis following treatment with culture supernatants diluted 1:100 and 1:1000.

At a dilution of 1:100 the effects of culture supernatants are not significantly different to those seen in undiluted samples. LCS supernatants continue to cause significant reductions in chemotaxis that are not significantly different to levels recorded in undiluted LCS supernatants.

At a dilution of 1:1000 approximately 160 LCS treated cells per field of view were observed migrating towards the attractant which is a significantly greater number than seen in both neat and 1:100 diluted sample (approximately 75 cells). Whilst results from LCS treated cells were significantly lower than those from MC and LC treated cells when incubated both neat and at 1:100, at a dilution of 1:1000 there were no significant differences between them. Similarly, at 1:1000 dilution LCS supernatants did not cause significant reductions in chemotaxis when compared to untreated controls.

The Effects of Proteinase K Digested Culture Supernatants on Chemotaxis Of Salmon Macrophages

Figure 7.4 shows the effects on macrophage chemotaxis of pre-treatment of culture supernatants with 0.5mg/ml proteinase K.

Without proteinase K treatment, LCS supernatants caused significantly reduced chemotaxis. MC supernatants did not affect rates of chemotaxis, whilst LC supernatants significantly reduced the ability of cells to migrate towards the attractant. However, the effect caused by the LCS supernatants were significantly greater than those caused by LC supernatants.

Following proteinase K digestion the rates of chemotaxis by cells treated with each culture supernatant were not significantly different to those of untreated macrophages. Similarly, there were no significant differences between the effects caused by each of the supernatant groups at each sample point.

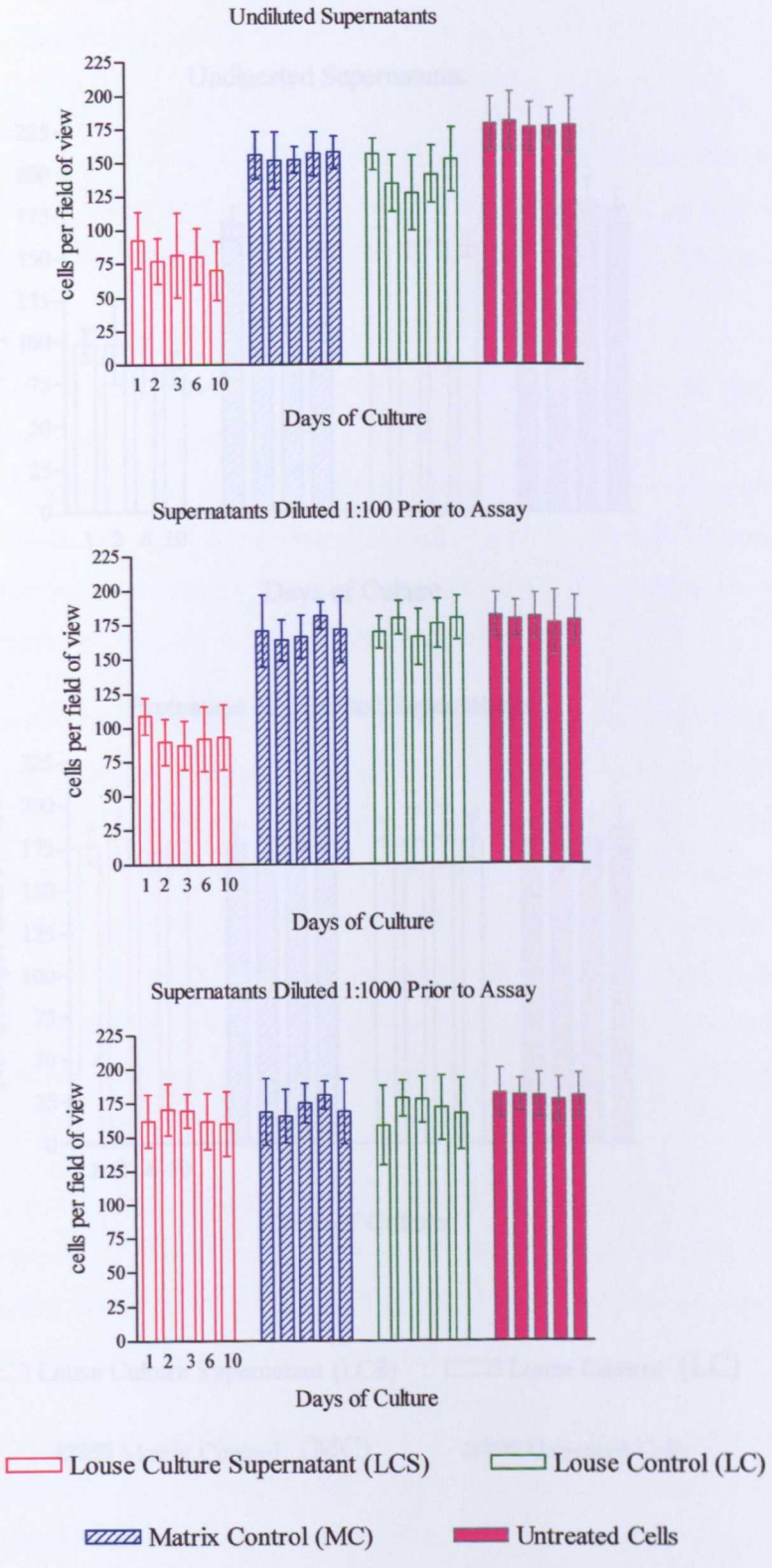


Figure 7.3

The effects of diluted culture supernatants on chemotaxis by salmon macrophages. Data represent the net movement of cells towards an attractant and are the mean (± standard deviation) calculated from 12 chemotaxis chamber observations per treatment. Treatments are presented graphically as groups with each bar representing a single treatment pertaining to a supernatant collected from one day of a copepodid culture cycle (in the order day 1,2,3,6 & 10).

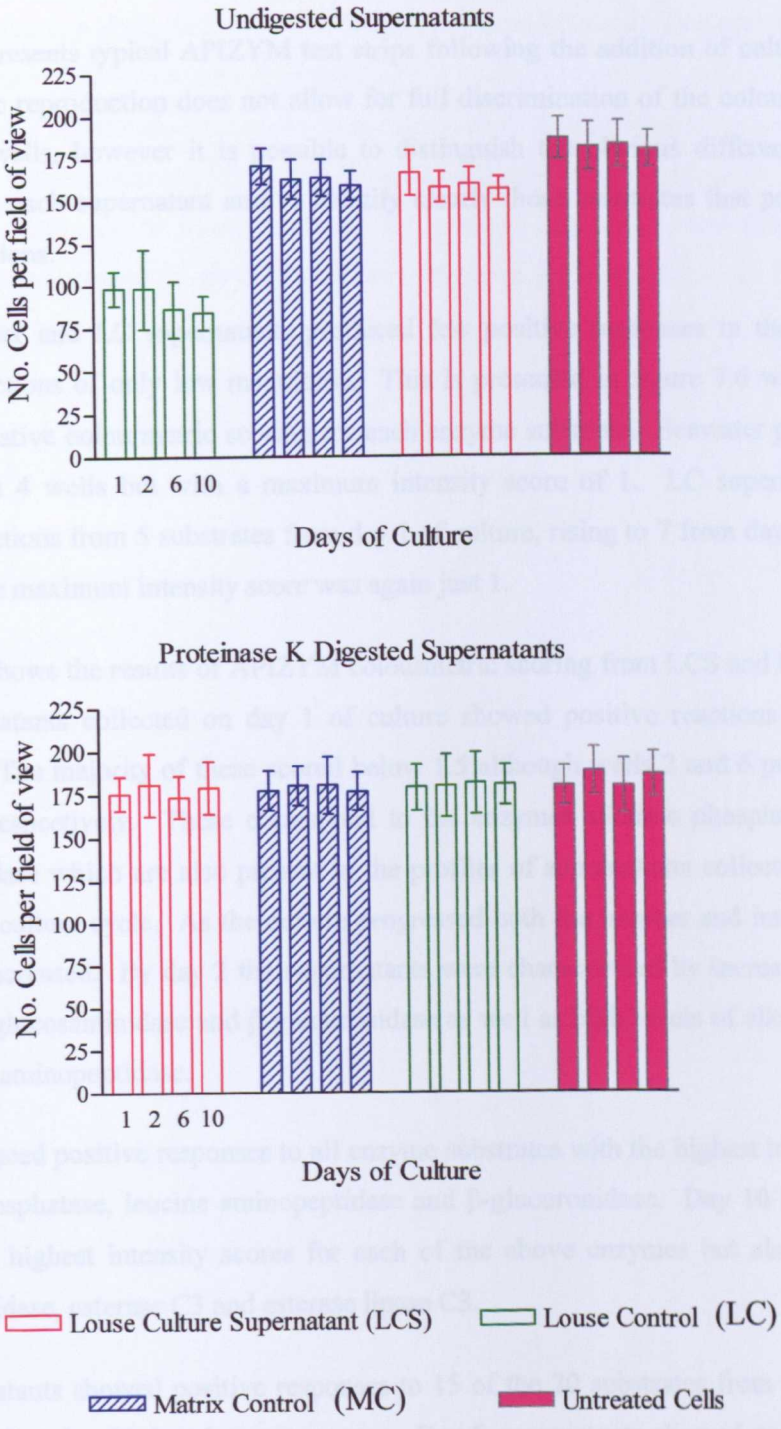


Figure 7.4

The effects of Proteinase K treated culture supernatants on chemotaxis by salmon macrophages. Data represent the net movement of cells towards an attractant and are the mean (± standard deviation) calculated from 12 chemotaxis chamber observations per treatment. Treatments are presented graphically as groups with each bar representing a single treatment pertaining to a supernatant collected from one day of a copepodid culture cycle (in the order day 1,2,3,6 & 10).

APIZYM Profiles of Culture Supernatants

Figure 7.5 presents typical APIZYM test strips following the addition of culture supernatants. Photographic reproduction does not allow for full discrimination of the colourmetric reactions in the test wells, however it is possible to distinguish the obvious differences between the responses to each supernatant and to identify clearly those substrates that produced the most intense reactions.

Both seawater and LC supernatants produced few positive responses in the test wells with positive reactions of only low magnitude. This is presented in figure 7.6 which displays the semi-quantitative colourmetric scoring for each enzyme substrate. Seawater produced positive responses in 4 wells but with a maximum intensity score of 1. LC supernatants produced positive reactions from 5 substrates from day 1 of culture, rising to 7 from day 10 supernatants, however, the maximum intensity score was again just 1.

Figure 7.7 shows the results of APIZYM colourmetric scoring from LCS and MC supernatants. LCS supernatants collected on day 1 of culture showed positive reactions in 12 of the 20 substrates. The majority of these scored below 1.5 although wells 2 and 6 produced scores of 2.5 and 5 respectively. These correspond to the enzymes alkaline phosphatase and leucine aminopeptidase which are also present in the profiles of supernatants collected on subsequent days of the culture cycle. As the culture progressed both the number and intensity of positive responses increased. By day 2 the supernatants were characterised by increased responses for N-acetyl-β-glucosaminidase and β-glucuronidase as well as high levels of alkaline phosphatase and leucine aminopeptidase.

Day 8 produced positive responses to all enzyme substrates with the highest intensity scores for alkaline phosphatase, leucine aminopeptidase and β-glucuronidase. Day 10 LCS supernatants showed the highest intensity scores for each of the above enzymes but also for N-acetyl-β-glucosaminidase, esterase C3 and esterase lipase C3.

MC supernatants showed positive responses to 15 of the 20 substrates from samples collected on day 1 although with low intensity scores. Day 8 supernatants showed positive reactions to all substrates but with a maximum intensity score of 2. There were slight increases in the intensity of reactions in day 10 supernatants with the highest scores for valine aminopeptidase and β-glucuronidase, but the profile at this time, as indeed at previous time points, is significantly different to that seen in LCS supernatants.

Figure 7.8 presents colourmetric scoring of whole copepodid homogenates from day 2 and day 6 post metamorphosis from nauplius I. Newly moulted copepodids showed positive responses

to only 7 enzyme substrates, but with significant responses to 4 of these; alkaline phosphatase; acid phosphatase; β-glucuronidase; N-acetyl-β-glucosaminidase. Homogenates from day 6 post-moult showed reaction to a greater number of enzyme substrates, but only at low levels. There appears to be a slight reduction in the concentration of N-acetyl-β-glucosaminidase and slight increases in valine aminopeptidase and Naphthol-AS-BI-phosphohydrolase. These profiles are significantly different to those produced by each of the culture substrates, although the copepodid homogenates do show comparable levels of β-glucuronidase to those measured in LCS supernatants.

Seawater

Louse Control Supernatant (LC)

Matrix Control Supernatant (MC)

Louse Culture Supernatant (LCS)

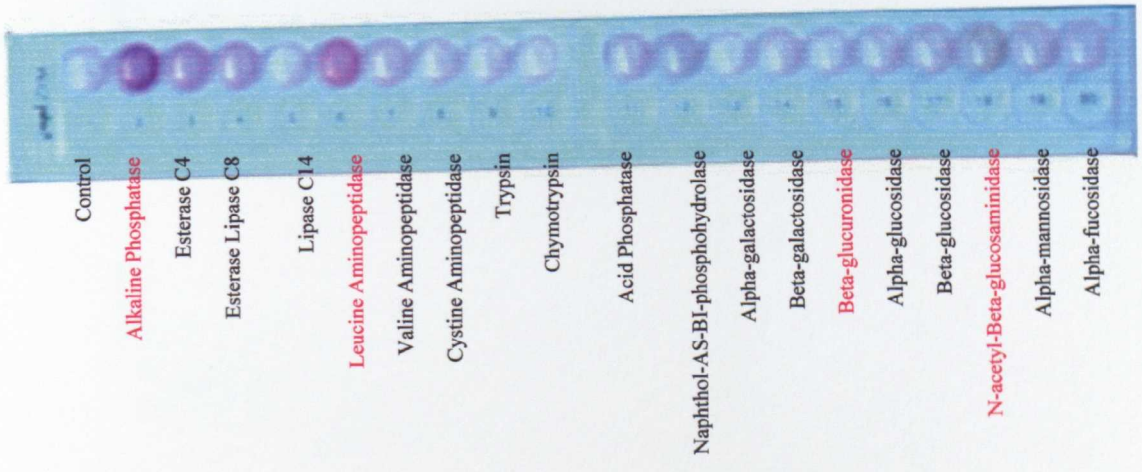


Figure 7.5

The enzymatic profiles of culture supernatants as determined using APIZYM test strips. Photographic reproduction of test results does not allow full visualisation of the colourmetirc reactions in all wells but does allow identification of those showing the greatest intensity of reaction. The nature of each test well is labelled with those producing the strongest reactions highlighted in red.

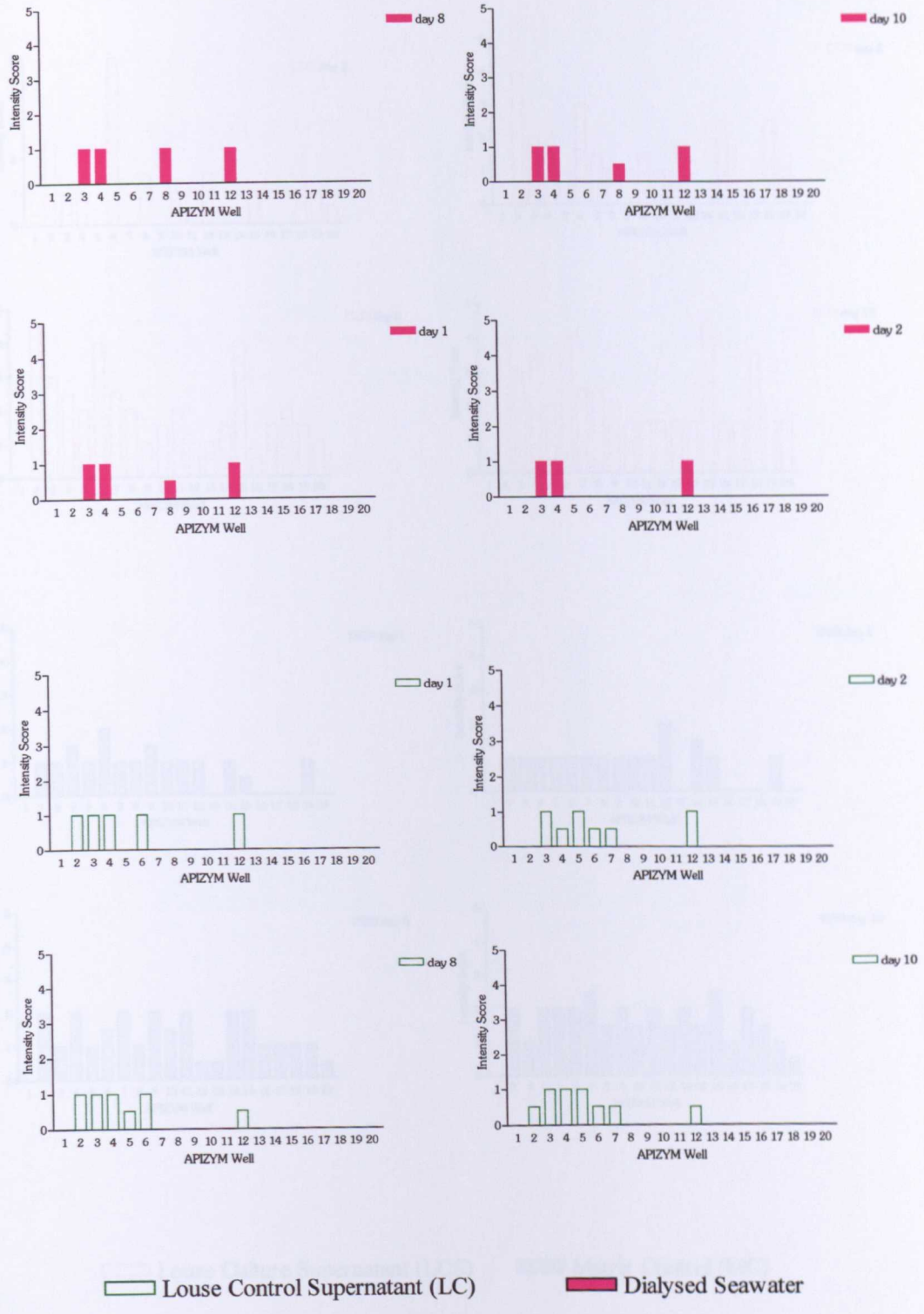


Figure 7.6

The intensity of APIZYM reactions following the addition of Louse Control (LC) and Seawater control supernatants to test strips. Data are mean of duplicate strips prepared for each supernatant at each of the 4 time points of culture cycles 2 & 3 and represent the mean colourmetric response of the APIZYM test wells compared to a standard intensity score chart (Biomerieux). The approximate enzyme concentration at each intensity is: 1 ~ 5nM enzyme/ml; 2 ~ 10nM/ml; 3 ~ 20nM/ml; 4 ~ 30nM/ml; 5 > 40nM/ml.

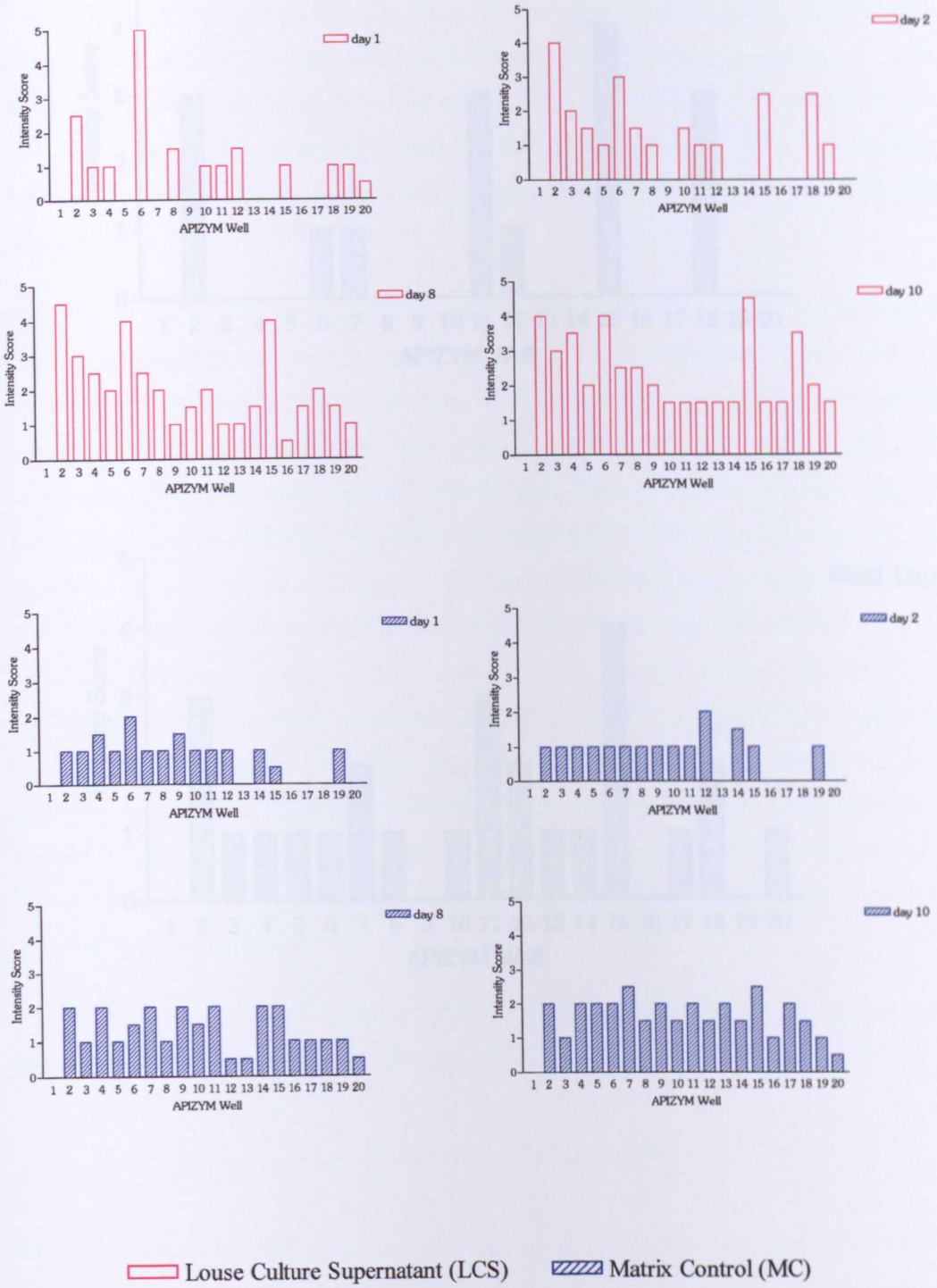


Figure 7.7

The intensity of APIZYM reactions following the addition of Louse Culture Supernatant (LCS) and Matrix Control (MC) supernatants to test strips. Data are mean of duplicate strips prepared for each supernatant at each of the 4 time points of culture cycles 2 & 3 and represent the mean colourmetric response of the APIZYM test wells compared to a standard intensity score chart (Biomerieux). The approximate enzyme concentration at each intensity is: 1 ~ 5nM enzyme/ml; 2 ~ 10nM/ml; 3 ~ 20nM/ml; 4 ~ 30nM/ml; 5 > 40nM/ml.

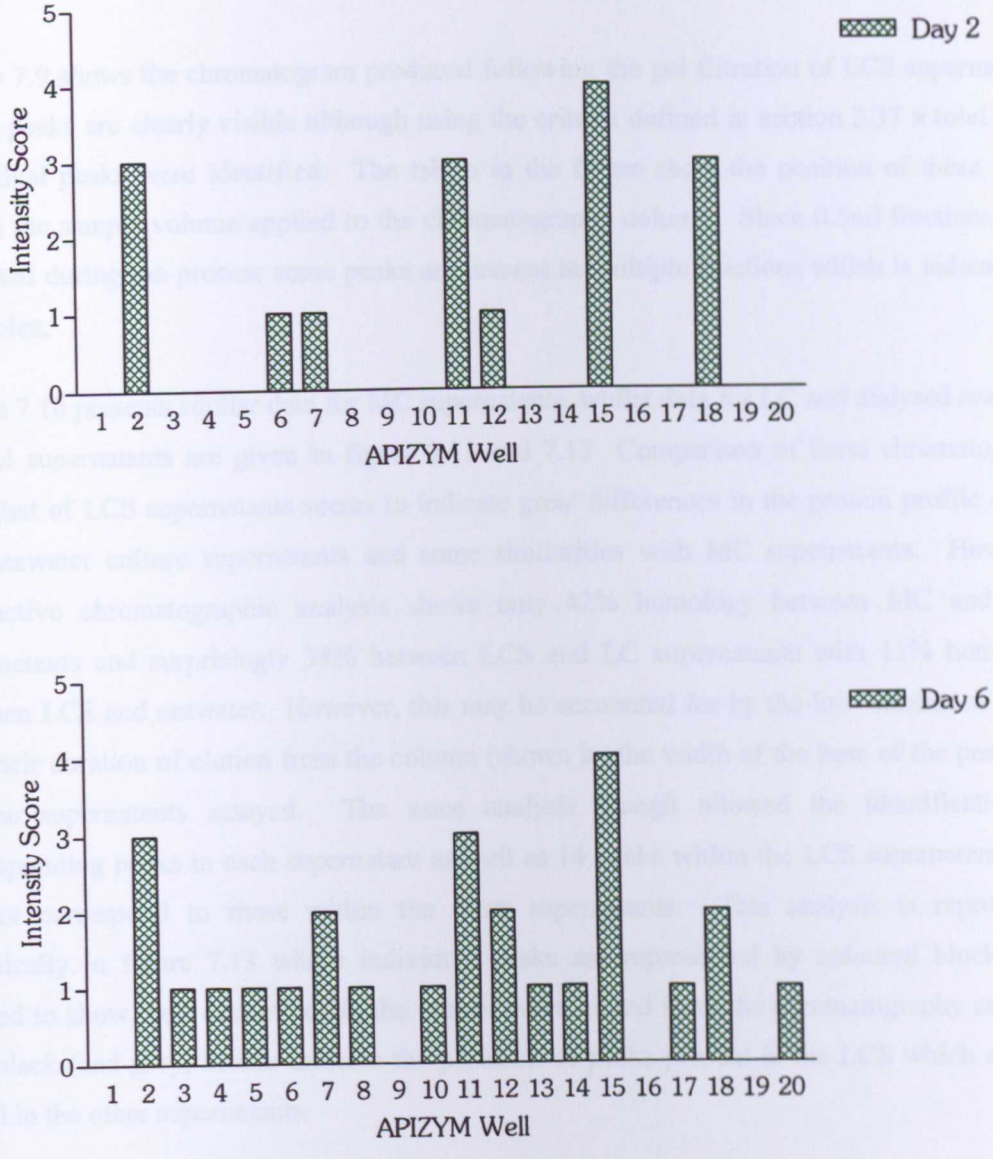


Figure 7.8

The intensity of APIZYM reactions following the addition of copepodid homogenates of animals collected 2 and 6 days post metamorphosis and maintained in seawater to test strips. Data are mean of duplicate strips and represent the mean colourmetric response of the APIZYM test wells compared to a standard intensity score chart (Biomerieux). The approximate enzyme concentration at each intensity is: 1 ~ 5nM enzyme/ml; 2 ~ 10nM/ml; 3 ~ 20nM/ml; 4 ~ 30nM/ml; 5 > 40nM/ml.

The Molecular Size of Culture Supernatant Components Determined By FPLC

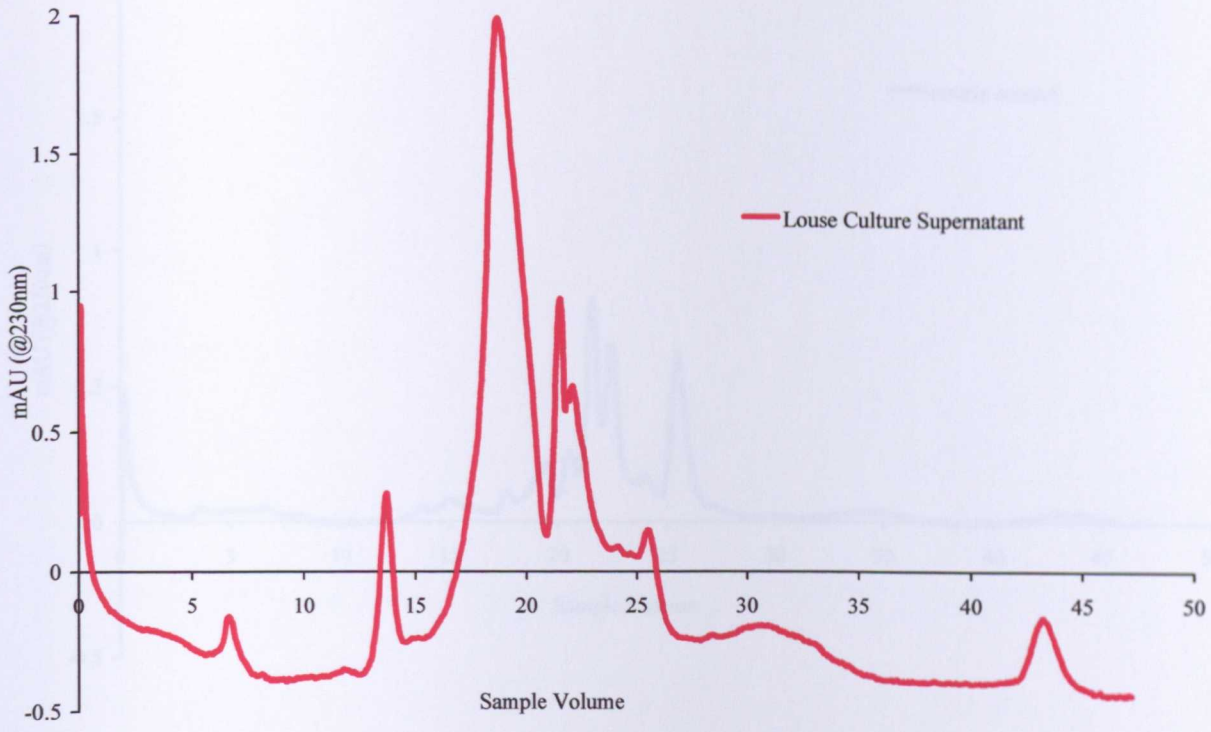
Figure 7.9 shows the chromatogram produced following the gel filtration of LCS supernatants. Seven peaks are clearly visible although using the criteria defined in section 2.37 a total of 34 individual peaks were identified. The tables in the figure show the position of these peaks within the sample volume applied to the chromatography column. Since 0.5ml fractions were collected during the process some peaks are present in multiple fractions which is indicated in the tables.

Figure 7.10 presents similar data for MC supernatants, whilst data for LC and dialysed seawater control supernatants are given in figure 7.11 and 7.12 Comparison of these chromatograms with that of LCS supernatants seems to indicate great differences in the protein profile of LC and seawater culture supernatants and some similarities with MC supernatants. However, subtractive chromatographic analysis shows only 42% homology between MC and LCS supernatants and surprisingly 38% between LCS and LC supernatants with 11% homology between LCS and seawater. However, this may be accounted for by the low number of peaks and their duration of elution from the column (shown by the width of the base of the peaks) in all the supernatants assayed. The same analysis though allowed the identification of corresponding peaks in each supernatant as well as 14 peaks within the LCS supernatants that do not correspond to those within the other supernatants. This analysis is reproduced graphically in figure 7.13 where individual peaks are represented by coloured blocks and aligned to show their occurrence in the sample when eluted from the chromatography column. The black (and grey) blocks indicate the positions of peaks present in the LCS which are not found in the other supernatants.

The calibration of the gel filtration column (section 2.37) allowed the calculation of the molecular weights of the proteins represented by these 14 peaks using the formula:

$$\text{Molecular weight (KDa)} = 10^{(\text{sample volume} - 22.467) / -4.80693}$$

The molecular weights of proteins found only within LCS supernatants are given in table 7.1. The majority of these were eluted from the gel filtration column close to the start of the process indicating larger proteins. These have been estimated as being between 150 and 2665 KDa. The remaining proteins and polypeptides have molecular weights below 22 KDa and down to values outside of the acceptable error margins for the apparatus.

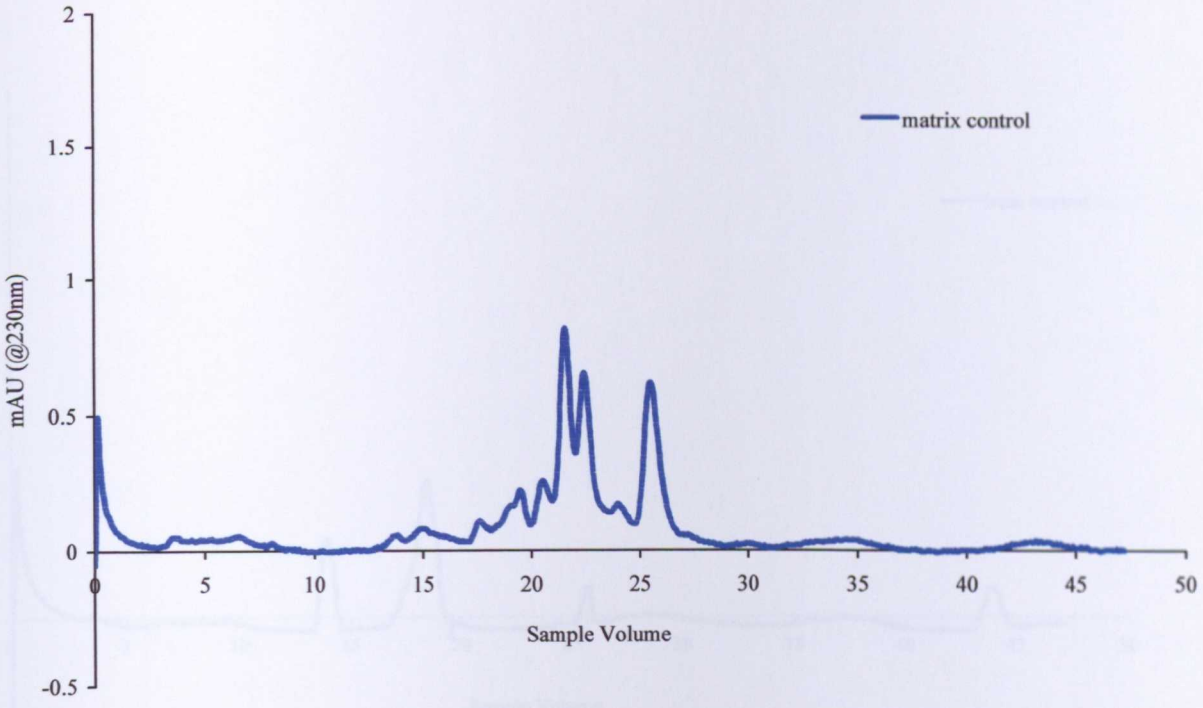


Peak No.	Sample Volume (ml)
1	4.0, 4.5
2	5.5, 6.0
3	7.0
4	7.5
5	8.5
6	9.0
7	10.0
8	11.5, 12.0
9	13.0, 13.5
10	14.0
11	15.5
12	16.0
13	17.5, 18.0
14	18.5
15	19.0, 19.5
16	20.0
17	20.5

Peak No.	Sample Volume (ml)
18	21.5, 22.0
19	23.0
20	23.5
21	24.5
22	25.0
23	25.5, 26.0
24	27.5
25	28.5, 29.0
26	31.0
27	32.0
28	34.5
29	36.0
30	42.0, 42.5, 43.0
31	43.5
32	44.0, 44.5
33	46
34	47.5

Figure 7.9

Chromatogram of the gel filtration of Louse Culture Supernatant (LCS). LCS samples from day 5 of culture cycles 1 & 4 were assayed and produced comparable chromatographic output. The tables indicate the number of peaks identified on the chromatograms and the sample volume at which these peaks occurred.

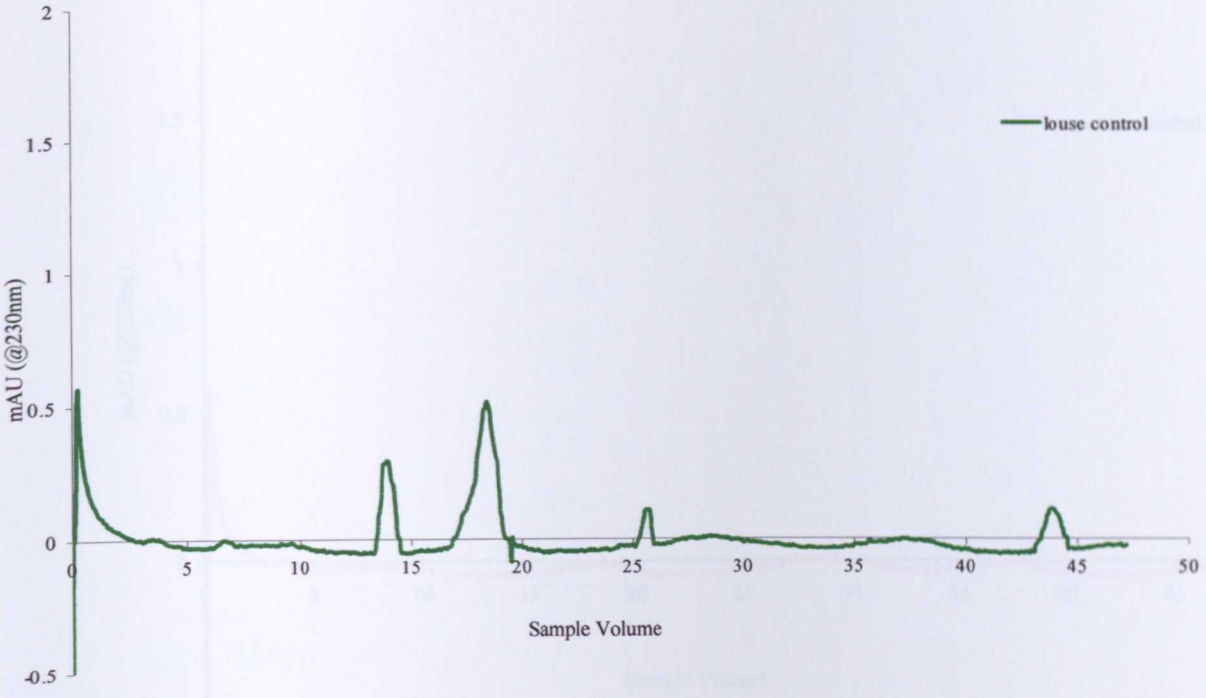


Peak No.	Sample Volume (ml)
1	4.0, 4.5
2	6.5
3	8.0
4	13.0
5	15.5
6	17.5, 18.0
7	19.0
8	19.5
9	20.5, 21.0
10	21.5
11	22.0

Peak No.	Sample Volume (ml)
12	23.0
13	24.0, 24.5
14	25.5
15	26.5
16	28.5, 29.0
17	31.5
18	33.0
19	35.0, 35.5
20	42.0, 42.5, 43.0
21	46.0
22	48.5

Figure 7.10

Chromatogram of the gel filtration of Matrix Control (MC) supernatant. MC samples from day 5 of culture cycles 1 & 4 were assayed and produced comparable chromatographic output. The tables indicate the number of peaks identified on the chromatograms and the sample volume at which these peaks occurred.

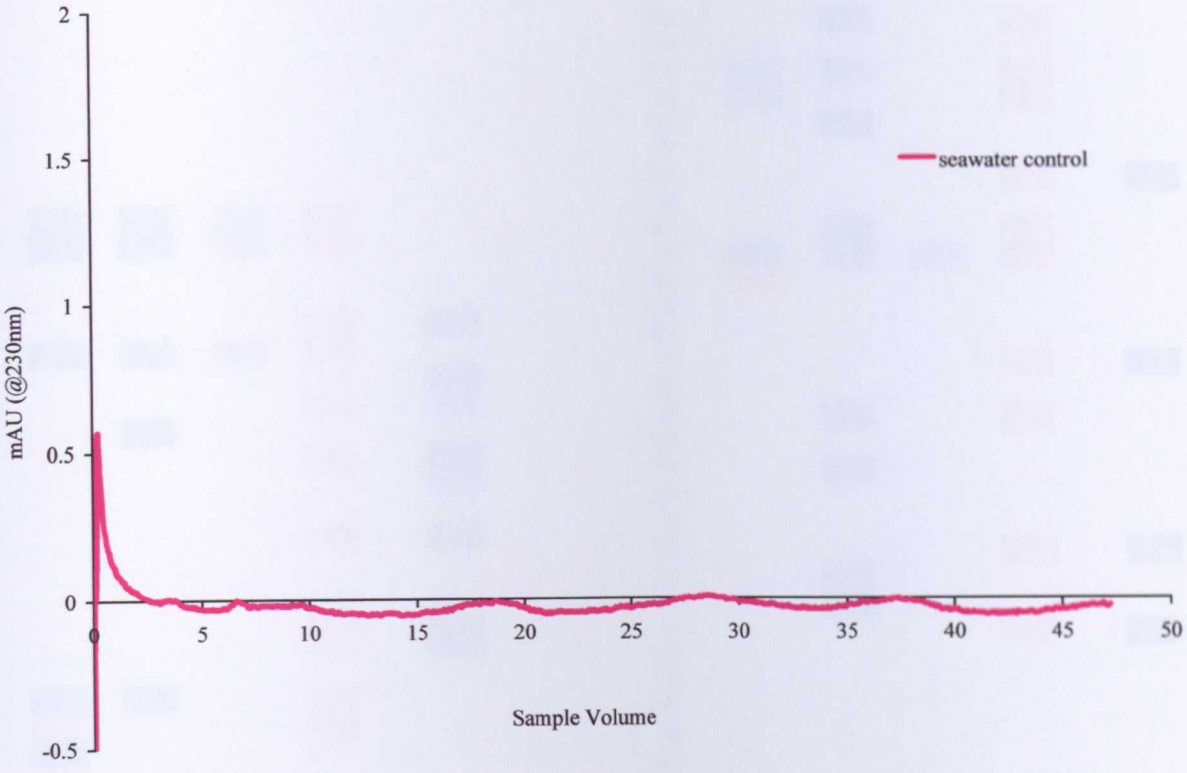


Peak No.	Sample Volume (ml)
1	4.0, 4.5
2	6.5
3	13
4	13.6
5	14
6	14.5
7	17
8	17.5

Peak No.	Sample Volume (ml)
9	18.0
10	18.5, 19.0
11	25.5, 26.0
12	29.0
13	29.5
14	42.0, 42.5, 43.0
15	43.5
16	44.0

Figure 7.11

Chromatogram of the gel filtration of Louse Control (LC) supernatant. LC samples from day 5 of culture cycles 1 & 4 were assayed and produced comparable chromatographic output. The tables indicate the number of peaks identified on the chromatograms and the sample volume at which these peaks occurred.



Peak No.	Sample Volume (ml)
1	4.0, 4.5
2	6.5
3	18.0
4	29.0

Figure 7.12

Chromatogram of the gel filtration of Dialysed seawater (culture control). Seawater samples from day 5 of culture cycles 1 & 4 were assayed and produced comparable chromatographic output. The table indicates the number of peaks identified on the chromatograms and the sample volume at which these peaks occurred.

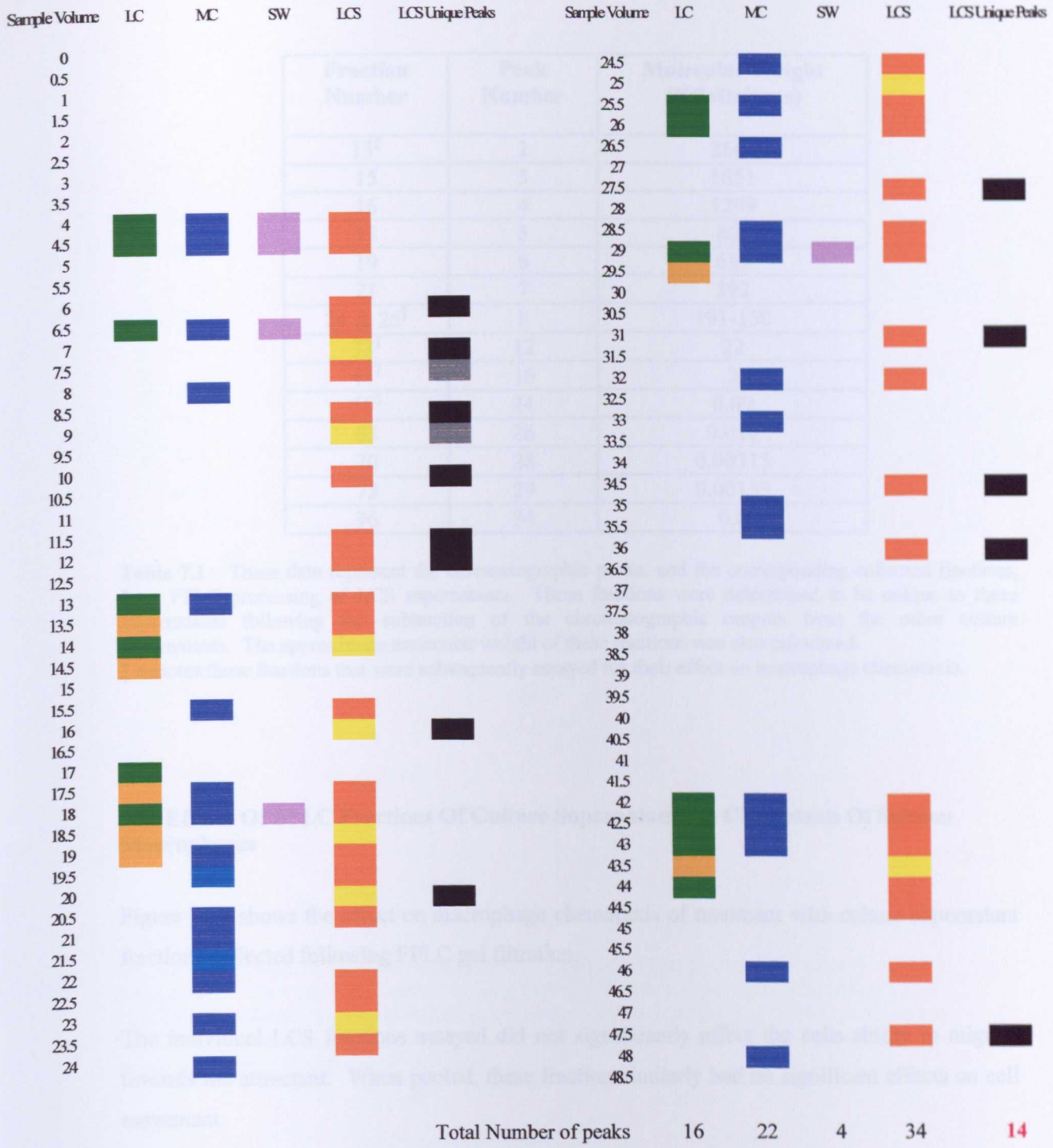


Figure 7.13

Graphical representation of subtractive chromatographic analysis. Blocks of colour represent individual peaks identified in each supernatant, a change in the colour within the same column indicates an additional peak occurring in close succession. The balck (and grey) blocks, under the column title 'LCS Unique Peaks', represent the position of 14 peaks which are present in the LCS supernatants but which are absent in the other supernatants assayed. These data are the mean of duplicate analysis of samples collected on day 5 of culture cycles 1 and 4.

Fraction Number	Peak Number	Molecular Weight (KiloDaltons)
13‡	2	2665
15	3	1651
16	4	1299
18	5	805
19	6	633
21	7	392
24 & 25‡	8	191-150
33‡	12	22
41‡	16	3
56‡	24	0.09
63	26	0.016
70	28	0.00313
73	29	0.00153
96	34	0.0

Table 7.1 These data represent the chromatographic peaks, and the corresponding collected fractions, from FPLC processing of LCS supernatants. These fractions were determined to be unique to these supernatants following the subtraction of the chromatographic outputs from the other culture supernatants. The approximate molecular weight of these fractions was also calculated.
‡ denotes those fractions that were subsequently assayed for their effect on macrophage chemotaxis.

The Effects Of FPLC Fractions Of Culture Supernatants on Chemotaxis Of Salmon Macrophages

Figure 7.14 shows the effect on macrophage chemotaxis of treatment with culture supernatant fractions collected following FPLC gel filtration.

The individual LCS fractions assayed did not significantly affect the cells ability to migrate towards the attractant. When pooled, these fraction similarly had no significant effects on cell movement.

Pooled fractions from both MC and LC supernatants did not effect the chemotaxic capabilities of salmon macrophages. None of the fractions assayed influenced the rate of chemotaxis below that recorded by untreated macrophages of approximately 175 cells per field of view.

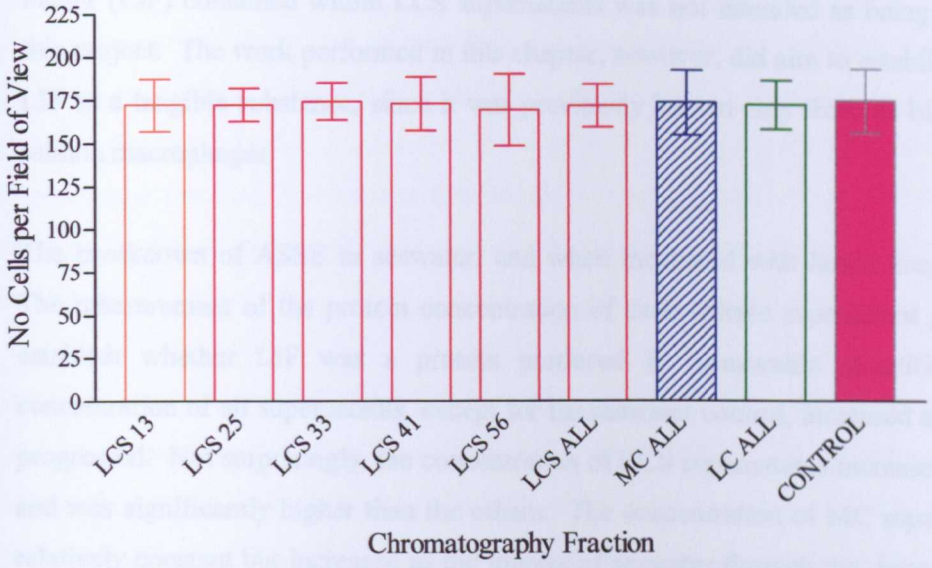


Figure 7.14

The effect of treatment with culture supernatant fractions on the chemotaxic ability of salmon macrophages. Data are mean number of cells moving towards a chemoattractant and represent data from 4 replicate chemotaxis chambers per treatment. Samples referred to as ALL represent equal volumes of the 14 unique LCS fractions, and equal volumes of all peak containing fractions identified in MC and LC supernatants.

DISCUSSION

An extensive and detailed investigation of the biochemical nature of Louse Immunomodulatory Factor (LIF) contained within LCS supernatants was not intended as being the main thrust of this project. The work performed in this chapter, however, did aim to establish the existence of LIF as a tangible substance, since it was previously known only from its biological effects on salmon macrophages.

The breakdown of ASSE in seawater, and when incubated with larval lice, liberates protein. The measurement of the protein concentration of each culture supernatant group will help to establish whether LIF was a protein produced in measurable quantities. The protein concentration of all supernatants, except for the seawater control, increased as the culture cycle progressed. Not surprisingly, the concentration of LCS supernatants increased the most rapidly and was significantly higher than the others. The concentration of MC supernatants remained relatively constant but increased as the ingress of seawater through the damaged surface layers caused the destruction of cells. Similarly, the concentration of LC supernatants was relatively constant but increased from around day 7 onwards, probably as a consequence of the death and degradation of the copepodids. The net difference between LCS supernatants and the control supernatants is approximately 0.1mg/ml during the first week of the culture cycles. Thereafter, the difference increases, probably as a result of the increasing mortality of copepodids from this point, and the liberation of proteins from their degradation. The net protein gain in the early stages of culture indicates the secretion or excretion of proteineacous material by copepodids cultured on the cellular substrate that was not produced whilst they were free-swimming (seen in figure 7.1a). Previous chapters have reported observations of copepodids feeding on the ASSE which suggests that additional protein input might be from the excretion of digested material. However, the metabolic requirements of arthropods for dietary protein to permit growth, in common with other animals would limit the quantities being excreted. This project has no measure of the protein intake of ASSE cultured copepodids and therefore no indication of the proportion that the net gain in protein in LCS of 0.1mg/ml forms as part of the total amount ingested. Whilst proteinaceous material in faeces would be expected, the additional protein in LCS may also be secreted by the copepodid, possibly to aid in pre-digestion of food in much the same way as some insects, for example, the Diptera. However, Jones *et al.* (1990) were unable to find histological evidence of external digestion by larval *L. salmonis* infecting Atlantic salmon. An alternative hypothesis, is that the additional proteins are immunologically

aggressive compounds produced by the infecting copepodid to enable effective parasitism. This was suggested from the work of Balasundaram et al. (1995), who reported elevated protein concentrations in the supernatants of copepodids maintained within their *in vitro* culture environment. However, there are problems of reproducibility with the method that these authors used and so their measurements have not been confirmed.

In this preliminary study, no further tests were performed to measure the occurrence of additional quantities of other major biochemical groups in LCS supernatants. A hypothesis was therefore proposed based around the measurement of additional protein that, it is this proteinaceous material that causes the immunological depression of salmon macrophages following incubation with LCS supernatants. Subsequent experiments were aimed at testing this hypothesis.

Culture supernatants were pre-treated prior to their incubation with salmon macrophages. In the first set of experiments, they were heated to 40°C and to 80°C, then incubated with macrophages, which were then tested for their chemotaxic responses. Heating to 40°C had no significant effect on the LIF (contained within LCS supernatants) -mediated reduction of chemotaxis, which was still significantly lower than that of the other, non-heated, culture supernatant treated cell populations. Heating to 80°C, however, had a significant negative effect on LIF activity/effectiveness. LIF treated cells still showed reduced chemotaxis, when compared to non-LIF treated cells, but the effect was significantly less than seen in un-heated LIF treated cells. This indicates that the LIF active component(s) are not heat stable.

In a second experiment, culture supernatants were diluted and then assayed for their action on macrophage chemotaxis. At a dilution of 1:100, there was no apparent effect on the ability of LIF to cause reduced chemotaxis. At 1:1000 however, its ability was significantly reduced and the rate of chemotaxis of diluted LIF treated cells was not significantly different to that of cells treated with the other (non-LIF containing) culture supernatants. Unfortunately, a more comprehensive dilution sequence was not used which would have provided the Inhibitory Concentration 50 (IC_{50}), the concentration of LIF that causes only 50% of its undiluted effect, and which would have enabled a degree of quantification of its activity. However, even without this information, it is possible to confirm LIF as a tangible substance since its effects can be diluted out and prevented by heating, and proves that its effects are not artefacts of the culture environment.

Inhibition of activity by heating and dilution are common properties of enzymes and other proteins which, coupled with the measured increase in protein within LCS supernatants, seems to indicate that the hypothesis on the protein nature of LIF may stand. Further evidence of this though was provided by the digestion of the protein within LCS by the endopeptidase proteinase K. Digested LIF-containing supernatants did not reduce the rate of macrophage chemotaxis, indicating the inactivation of the immunomodulatory component by the enzyme. Whilst proteinase K is a broad-spectrum protease which shows very little cleavage specificity, it has not documented effects on non-proteins (Worthington, 1988). This would seem to confirm LIF as a protein.

However, an alternative explanation is possible, which is that LIF is only active in the presence of a protein or amino acids, and that it itself is not a protein. There is limited evidence amongst arthropod parasites that supplementary compounds are required to activate immunomodulatory mechanisms. In a review by Wikel *et al.* (1996), passing comment is made of early theories that suggested that immunosuppressive compounds of ticks required the presence of host blood to encourage their secretion and activity *in vitro*. This has subsequently been shown not to be the case, since ticks commonly secrete saliva, containing immunosuppressants, spontaneously and without association with host tissues. However, the survival and feeding of the female of the tick *Rhipicephalus appendiculatus* is severely impaired in the absence of immunoglobulin-binding-proteins (IGBPs) that are secreted by the male tick (Wang, *et al.*, 1998). The tick employs IGBPs as defence against the host immune response. IGBP-MC, specifically, is released by the male in the presence of a female tick that stimulates her production of IGBPs and other immunomodulators. In the absence of secreted immunomodulators the female ticks parasitic ability is significantly reduced which ensures her reliance on the male and guarantees the male a mate. The biochemistry of the interaction between secreted IGBP-MC and the activation of the females immunomodulators is not fully understood, but the possibility of the biochemical requirement for these proteins to activate the immunomodulatory factors of the female cannot be ignored. Similarly, *Schistosoma mansoni* cercariae require a specific fatty acid profile to enable successful penetration of host tissues without the involvement of host immune defences (Fusco *et al.*, 1986). This parasite also produces heat shock proteins that act as immunomodulatory factors by stimulating an immune response against non-functional epitopes on its own surface to deceive host defences, but only following penetration of the host (Shinnick, 1991). The trigger for the activation of these mechanisms is not fully known, and as well as changes in environment, identification of host receptor molecules and stimulation by the host immune system, the involvement of fatty acids in the transformation is also speculated (De Jong-Brink, 1995).

FPLC analysis of culture supernatants was used to identify the differences in the protein profiles of the culture supernatants; to distinguish the proteins that may constitute LIF; to collect these proteins; and the to test their individual effects on salmon macrophage immunomodulation. This appeared the most relevant method of establishing whether LIF was actually a protein in nature, or if it only required the presence of proteins to function. Following gel filtration chromatography, 34 components of LCS supernatants were isolated, 22 from MC supernatants, 16 from LC supernatants, and 4 from seawater. The comparison of the chromatograms generated by this method allows clear identification of the differences between each supernatant. However, the more accurate method of subtractive chromatographic analysis allowed the subtraction of corresponding peaks in each supernatant and identified 14 components in LCS supernatants that were not present in any of the other culture supernatants. The remaining LCS components were shared by MC and LC supernatants and were likely derived from the breakdown of ASSE in seawater and the excretion of metabolites by free-swimming copepodids.

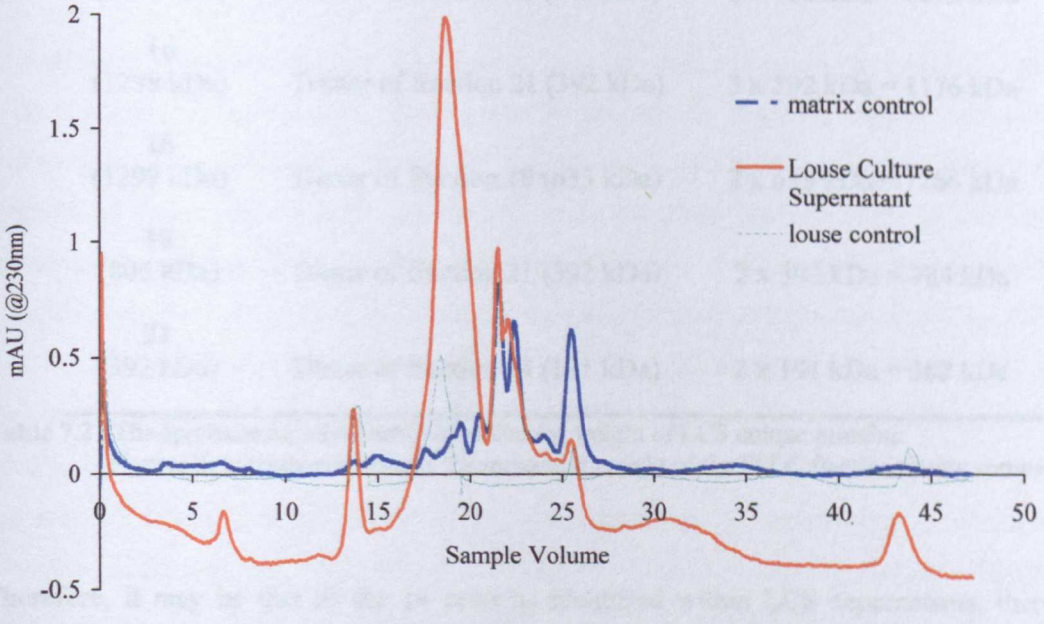


Figure 7.15 Chromatograms produced from the gel filtration of sea louse culture supernatants (LCS) and culture control supernatants (MC & LC). Reproduced from figures 7.9, 7.10 & 7.11.

The calibration of the gel filtration column allowed the determination of the molecular weights of these components that ranged from large globular proteins at 2665 kDa to peptides whose size was not accurately predictable using the methods applied in this study. The majority of these proteins are large and were eluted from the column early in the process and so techniques can be used to more accurately calculate the molecular weight of each component protein. This will also establish whether the fractions were indeed 14 different proteins, or whether the larger proteins are polymers of smaller ones. However, whilst not being able to predict this accurately, the relationships between the molecular weights of these proteins can be suggested from the data collected from the method used in this study, and are given in the table below.

Fraction No.	Relationship	Explanation
13 (2665 kDa)	Dimer of fraction 16 (1299 kDa)	2 x 1299 kDa = 2598 kDa
13 (2665 kDa)	Tetramer of fraction 19 (633 kDa)	4 x 633 kDa = 2532 kDa
15 (1651 kDa)	Dimer of fraction 18 (805 kDa)	2 x 805 kDa = 1610 kDa
16 (1299 kDa)	Trimer of fraction 21 (392 kDa)	3 x 392 kDa = 1176 kDa
16 (1299 kDa)	Dimer of fraction 19 (633 kDa)	2 x 633 kDa = 1266 kDa
18 (805 kDa)	Dimer of fraction 21 (392 kDa)	2 x 392 kDa = 784 kDa
21 (392 kDa)	Dimer of fraction 24 (191 kDa)	2 x 191 kDa = 382 kDa

Table 7.2 The approximate relationship in molecular weight of LCS unique proteins. Figures in parentheses indicate the molecular weight of the FPLC fractions being compared

Therefore, it may be that of the 14 proteins identified within LCS supernatants, there may actually be only 4-5 individual proteins of smaller molecular weight, each of which constitutes a sub-unit of the other proteins collected.

L. salmonis infecting Atlantic salmon have been shown to secrete a series of serine proteases of the molecular weight range 17-22 kDa detected in the fish's mucus (Firth *et al.*, 1998; Firth *et*

al., 2000). They identified these proteins as being trypsin-like, and concluded that the lack of recognition of them by a monoclonal antibody to Atlantic salmon trypsin confirms their louse origin. The authors speculated on their role in external digestion and in evasion of host immune responses but were unable to produce supporting evidence for these hypotheses. However, the production of low molecular weight proteins by other arthropod parasites is better documented. Cross *et al.* (1993) identified a series of proteins in the saliva of the blackfly, *Simulium vittatum,* in the range 26-67 kDa that reacted with antibodies from mice immunised with the salivary gland extracts of the parasite. Whilst the saliva of this species is immunosuppressive in mice, no direct link has yet been established between these proteins and the suppressive effect. However, Ribeiro (1987) confirmed the immunosuppressive effects of a 49kDa protein isolated from the saliva of the tick *Ixodes dammini.* This substance was seen to inhibit the activation of the alternative complement pathway by preventing C3 hydrolysis and the deposition of C3b and C5b on the activating surface. This salivary factor was found to be effective in human, rat, mouse and guinea pig hosts. Other tick secreted proteins that appear conserved throughout the ixodid families, and which have comparable effects in multiple hosts include a 65 kDa anticoagulant protein first identified in the salivary extract of *R. appendiculatus* (Limo *et al*, 1991) and a 5 kDa protein that inhibits lymphocyte blastogenesis and reduces the responsiveness of lymphocytes to mitogens, *in vitro* (Urioste *et al*, 1994).

The secreted immunomodulatory factors of the schistosomid trematodes are probably the most widely studied owing to their involvement in the serious parasitic disease schistisomiasis (bilharzia). They are known to employ a range of arachidonic acid metabolites, primarily eicosanoids such as prostaglandins, with well documented suppressive effects on host immunity (reviewed by de Jong-Brink, 1995). However, they also produce an array of proteins and polypeptides that cause both localised and systemic immunodepression. *Trichobilharzia ocellata,* for instance, secretes a 40 kDa protein that specifically suppresses the phagocytic ability of haemocytes of the freshwater snail *Lymnaea stagnalis,* but which has no effect on the alternative host species *Planorbaris corneus* (Nunez *et al.*, 1994). Similarly, *S. mansoni* secretes a 22 kDa protein that inhibits protein secretion by haemocytes of *Biomphalaria glabrata* specifically (Lodes & Yoshino, 1990). In addition to these species specific proteins, Schistosomes also produce 2 protein, one of 30 kDa and one of 10 kDa, that down regulate haemocyte immunological activity in snail hosts which is demonstrated by Nunez *et al.* (1994) by the proteins inhibition of the cells bacterial killing ability. As mentioned previously in this discussion, *S. mansoni* also produces the heat shock proteins HSP60 and HSP70, where the classification is based on molecular weight (60 kDa and 70 kDa respectively). As well as their intracellular involvement in the management of peptides within the schistosomes cells, they are

expressed on their surfaces (Shinnick, 1991). HSPs are highly antigenic and amongst the dominant antigens recognised by the host immune response (Shinnick, 1991). Their expression at the parasite surface serves as an immunological 'smoke screen' that encourages an immune response directed towards these epitopes to divert attention from other, function epitopes.

It seems consistent that parasite-secreted immunomodulatory proteins are small, and in the range 5 kDa to 70 kDa in the examples reviewed. If this were applied to the results from FPLC analysis of the LCS fractions then the reasoning, that the larger components are polymers of several smaller proteins, seems accurate. Further speculation on either the number or the molecular weights of the active LIF proteins is not appropriate without further investigation, and it is sufficient at this stage to simply report the findings of the preliminary analyses to facilitate future study. However, the immunomodulating activity of individual protein fractions, which might consequently indicate the size range of active proteins, was measured by the effects of selected fractions on salmon macrophage chemotaxis. Five fractions were tested, as well as a sample containing equal volumetric proportions of each of the 14 fractions, but these each failed to demonstrate any immunomodulatory effects. The results were probably to be expected, since each fraction is eluted from the gel filtration column in buffer, which dilutes the original sample (100µl) 5000 times. As seen in figure 7.3, a dilution of 1:1000 was sufficient to prevent LIF activity in the native, unfractioned LCS supernatants, and so the lack of activity in the fractions is not a surprising result.

Whilst LIF has demonstrated immunomodulatory activity against macrophages, it should still be considered that this may be a coincidental effect caused by the secretion/excretion of non-aggressive metabolites from cultured copepodids that may not stimulate the same immunological response *in vivo*. APIZYM test strips were used to provide profiles, of responses to 19 common enzyme substrates, of each culture supernatant. These tests again highlighted the significant differences between the LCS supernatants and the control supernatants. The LCS generated responses to most of the enzyme substrates, but also produced significant reactions to the substrates for alkaline phosphatase, leucine aminopeptidase, N-acetyl-β-glucosaminidase and β-glucuronidase, as well as elevated responses for C4 and C8 esterases. These same enzymes were present in the matrix control supernatants, which indicates their involvement in cellular metabolism, but were not recorded in the quantities seen in the LCS supernatants. Whilst this may simply be a consequence of the more rapid degradation of ASSE through copepodid activity, and the subsequent release of these enzymes from damaged cells, there may be alternative explanations that are worth exploring.

Alkaline phosphatase is a common metabolic enzyme, and its presence in the profile of LCS can be easily explained by its release from damaged cells within the degrading ASSE. C4 and C8 esterases are digestive enzymes found in the alimentary canal of the mite *Psoroptes cuniculi* (Nisbet & Billingsley, 1999), and as a component of salivary gland extract of the cattle tick *Boophilus microplus* (Kerlin & Hughes, 1992). These authors however, established its digestive, rather than immunosupressant role. Leucine aminopeptidase is a proteolytic enzyme which is produced shortly following ingestion of food by *B. microplus* and *P. cuniculi*, and other arthropods, particularly following the ingestion of blood (Kerlin and Hughes, 1992; Nisbet and Billingsley, 1999; Stewart *et al.*, 1991). It is reasonable to assume that the presence of these enzymes within copepodid culture supernatants is indicative of digestion and further evidence of louse feeding within culture, and the suitability of ASSE to their maintenance. High levels of leucine aminopeptidase were also reported from the alimentary canal of *L. salmonis* by Roberts *et al.* (1999), as were high levels of N-acetyl-β-glucosaminidase. These authors attributed them with having a digestive function.

N-acetyl-glucosamine is the major constituent of chitin which is hydrolysed by the enzyme N-acetyl-β-glucosaminidase following apolysis, during insect moulting (Chapman, 1983). It is also found in the muscle and gills of crustaceans, but most commonly, and in the highest concentrations, in the gut (Molodstov & Vafina, 1972). Its occurrence in LCS supernatants is more than likely associated to excretion and the gut anatomy of *L. salmonis*, hence its discovery in the gut of the adult louse (Roberts *et al*, 1999). The mid-gut of insects, and *L. salmonis* copepodids (*pers. comm.* Dr. J. Bron), is lined by a peritrophic membrane/complex which is composed of chitin (Chapman, 1983). N-acetyl-β-glucosaminidase is involved in the reabsorbtion of this membrane and its subsequent secretion by cells of the mid-gut during growth and moulting, and in its maintenance following abrasion by solid food, for instance. As a consequence it is probable that quantities of this enzyme are therefore excreted in association with the peritrophic membrane which, as well as lining the gut, also coats the excreted faeces of both insects and *L. salmonis*.

Alternatively, the presence of chitin hydrolysing enzymes in LCS supernatants might be indicative of animals preparing to moult. No histological evidence was collected to determine ultrastructural changes in the cuticle of cultured copepodids, but the presence of these enzymes and the observations of copepodid growth may be indicative of moulting physiology. In this case, the occurrence of N-acetyl-β-glucosaminidase in LCS supernatants might be a result of the leaching of the enzyme from the cuticle, the permeability of which may increase during the early stages of moulting. However, there is no direct evidence of this.

It is apparent, therefore, that much further work is necessary before the enzymes, identified by APIZYM techniques, and the unique proteins identified by gel filtration chromatography, contained within LCS supernatants can be assigned definite roles. Their digestive involvement is of interest in investigating the physiology of *in vitro* cultured copepodids; in examining feeding; and in determining whether animals are preparing to moult; examining the processes surrounding moulting; and identifying the biochemical and physical requirements to stimulate moulting. The biochemical properties of the 14 LCS unique are of equal interest in the exploration of the immunomodulatory abilities of *L. salmonis* larvae and the possible incorporation of these active components into anti-louse pharmaceuticals or vaccines. This preliminary investigation of the nature and functions of cultured copepodid products provides a starting point for this future work.

Bibliography

Chapter 8

GENERAL DISCUSSION

In recent years, sea lice infections have become the most significant disease of farmed salmon in Scotland. They have also been implicated in the decline in wild sea trout and Atlantic salmon stocks in the northern Atlantic (Birkeland & Jakobsen, 1997). Research has concentrated, to a greater extent, on their biology and on methods for their control. Attempts at vaccination against *L. salmonis* using 'hidden' gut antigens, has proved largely unsuccessful thus far (Raynard *et al.*, 1994). Although the specific approaches to vaccine development used in these studies have been criticised, immunological control is recognised as the most appropriate method for the future control of this disease (Woo, 1997).

This current project was built, primarily, around the observations of Johnson & Albright (1992) that Atlantic salmon are more susceptible to *L. salmonis* infection than other salmonids, and that the parasite is successful on this species because of the hosts' inability to develop an effective cellular immune response toward the parasites. Stimulation and exploitation of host cellular immunity has been widely accepted as a realistic avenue of study for the management of arthropod parasites of livestock animals by the same authors who were originally successful with the 'hidden gut antigen' approach to the control of the same parasites (Inokuma *et al.*, 1993; Willadsen, 1987; Willadsen *et al.*, 1989; Willadsen & McKenna, 1991).

Many parasites have evolved mechanisms of manipulating host cellular responses by the production of immunosuppressive compounds (De Jong-Brink, 1995; Wikel *et al.*, 1996). In most hosts the cellular immune system is highly effective, and without exogenous modulation, would produce a rapid response that would kill the parasite, or prevent its infection. Atlantic salmon are unable to mount an effective cellular response to *L. salmonis* infection, which has led to speculation on the active immunosuppression of the host by the parasite (Johnson & Albright, 1992). This thesis aimed to investigate the basis of this speculation by collecting sea louse 'product' (excretory and/or secretory) and testing their effects on salmon macrophages. The anticipated complications of collection of these products from infected fish stimulated the development of an *in vitro* substitute 'salmon skin'. The work presented in the preceding chapters presents results both of the development and validation of the ASSE substrate, and of the collection, testing and characterisation of sea louse products, and it is these two themes that will be explored and summarised in this chapter.

The *in vitro* culture of parasitic organisms is largely limited to intracellular and micro-parasites, and of these, the successful culture of each developmental stage is not always possible (Smyth, 1990). The culture of *L. salmonis in vitro* was first attempted by Balasundaram *et al.*(1995) with limited reproducible success. Toovey *et al.* (2000) reported a simple tissue culture system that is capable of encouraging short-term association of copepodid larvae of *L. salmonis* with an Atlantic salmon cellular substrate, but whether this system constitutes 'culture' is debatable. The requirement for a robust cellular substrate, displaying the appropriate chemical and physical markers for louse recognition that is maintainable in seawater, presents a potentially insurmountable task. The original aspirations of the current project would indeed have been unachievable within the scope of this type of study. However, following the redefinition of the projects aims, successful larval culture was achieved. The ASSE constructed by this project, as well as having applications in the maintenance of sea lice, represents an organotypic system capable of encouraging cellular organisation, re-differentiation cultured cells functions, and of extended stability and growth in a seawater medium. It has the potential to be a remarkable innovation, which, though requiring additional development, lends itself to numerous applications. The advances in the understanding of mammalian integumental systems and responses, as a direct consequence of the development of human living skin equivalents (LSE) in the late 1970s, are significant and led directly to the creation of tissue engineering as a stand-alone scientific field (Phillips, 1993; Arnst & Carey, 1998; Eaglstein & Falanga, 1997). The suggestion is not that ASSE will impact in the same way, but within the fields of fish and lower vertebrate research its applicability, or least the techniques and reasoning behind it, could generate significant novel research. However, further, significant investigation of it as a 'tissue' is firstly required. In the same way that LSEs were extensively validated for their similarities to *in vivo* human skin (van de Sandt *et al.*, 1997), ASSE should be investigated to determine its comparative biochemistry, histology, cell receptor expression and tensile properties. At this stage of its development, these characteristics are likely to be very different, especially since there still exist significant differences between normal human skin and LSE, even after 2 decades of development. In spite of these differences though, LSE responses have been accurately correlated to the corresponding responses *in vivo* and are now used extensively as replacements for live animal models in, for example, chemical irritancy testing (van de Sandt, *et al.*, 1997).

In the present study, ASSE was used as a substrate for the maintenance of *L. salmonis*. Attempts at encouraging metamorphosis of copepodids settled on the substrate were unsuccessful. ASSE did, however, permit the extended maintenance of copepodid larvae for, on

average 5 days longer than they would have normally persisted when incubated without an appropriate cellular substrate. Furthermore, cultured copepodids were observed feeding on the substrate, performing 'normal' behaviour, and growing whilst maintained on ASSE. Copepodids are recorded feeding on their salmonid hosts prior to moulting (reviewed by Pike and Wadsworth, 1999), but metamorphosis by the copepodids feeding on ASSE did not occur. The likely absence of chemical and physical cues within ASSE that are required to stimulate metamorphosis, are discussed in chapter 5. In addition to the possible requirement for exogenous stimulation from the culture surface, it may also be that there is a need for the reception of additional stimuli whilst the copepodid is still free-swimming, or indeed, whilst it is at the naupliar stages. Rogers (1960), reviewing the *in vitro* culture of nematodes, hypothesised that nematodes in their natural environment have a system of, what he described as, "internal secretions" that regulated the timing of developmental events. Nematodes reared within the laboratory were less able to develop when incubated both *in vitro* and within host animals because, using his terminology, they lacked the internal secretions that the animal in its natural environment possessed. Comparison of the developmental success of the same stages of the two parasite forms, the 'wild-type' and the laboratory reared, showed increased developmental success of the 'wild-type', even when cultured in an *in vitro* cellular system. This system was originally thought to be deficient in required elements, and so responsible for the lack of developmental by laboratory nematodes. It may be argued then that laboratory hatched nauplii of *L. salmonis* do not receive the required stimuli that results in the eventual successful attachment and metamorphosis of the copepodid stages, but that in other ways they are normal. Since supplementation of ASSE did not stimulate copepodid metamorphosis an interesting hypothesis is that ASSE is a suitable substrate for the normal development of *L. salmonis* larvae, but that the copepodids used in the culture assays are incapable of normal developmental progression. This is obviously not the case since laboratory hatched copepodids have been extensively used in fish infectivity studies where they have developed normally. However, taking 'wild-type' copepodids, that have received a full and normal set of stimuli, and then introducing them to the ASSE would be an insightful experiment. It may be that nothing happens, in which case it would confirm that ASSE is biochemically, or physically deficient. Alternatively, the copepodids may be sufficiently stimulated that attachment to ASSE, or even non-cellular surfaces, occurs and metamorphosis follows.

The lack of normal development did not, however, divert the aims of the project significantly and the encouraging evidence of normal-type interactions with the ASSE (feeding, growth, settlement and survival) permitted progression to investigating the study's other objectives.

Supernatants collected from copepodid culture with ASSE caused the immunodepression of the chemotaxic and phagocytic defence responses of salmon macrophages. Comparable responses have been reported in other arthropod parasites and have been primarily associated with the secretion of PGE_2 by the parasites (Wikel *et al.*, 1994; Wikel, *et al.*, 1996). Other immunosupressive effects have been caused in the hosts of arthropod parasites that have been attributed to low molecular weight proteins that tend to interfere with the host non-specific humoral immune defences (Limo *et al.*, 1991; Urioste *et al.*, 1994). This current project did not investigate the lipid biochemistry of culture supernatants and so was unable to establish the presence of arachidonic acid metabolites such as prostaglandins that might be responsible for the depressive effects on salmon macrophages. However, protein analysis did identify 14 proteins in the LCS supernatants within the range <1 kDa to >2000 kDa, that, when inactivated by heating, dilution and incubation with proteinase K, caused no immunomodulation. The larger proteins identified are probably polymers of the smaller proteins, as discussed in chapter7, so that the bioactive component, designated Louse Immunomodulatory Factor (LIF), may consist of only 4-5 proteins with molecular weights <200 kDa. Immunosuppressive proteins produced by parasitic arthropods (ticks) (Wikel, *et al.*, 1996) and digeneans (schistosomes) (Nunez *et al.*, 1994) tend to be low molecular weight proteins, less than 70 kDa in schistosomes (Nunez, *et al.*, 1994). In arthropods, though, these proteins are more normally associated with the modulation of non-cellular immunity such as interfering in the complement cascade (Ribeiro, 1987). In schistosomes, on the other hand, these proteins are associated with interference with the defensive and metabolic capabilities of the haemocytes of the parasites' snail hosts (Nunez, *et al.*, 1994).

The expectation may be that *L. salmonis* immunomodulatory mechanisms should more closely resemble those employed by ticks, since they share a more recent common ancestry, rather than those of the schistosomes. However, the full range of active molecules from these and other parasitic groups, and the effects they have on the host, are not fully identified, and future research may reveal compounds that are conserved and shared by parasitic species from all phyla. Similarly, the work presented in this thesis is preliminary and further investigation may identify additional compounds, proteins or otherwise, that have effects against other functional arms of the salmons immune system. For instance, this thesis speculates, in chapter 6, that the mode of action of LIF may be directed towards the inhibition, or confusion of intra- and/or intercellular communication, which are regulated in defensive cells by cytokines. A variety of cytokines are also essential in stimulating macrophage activation and immune functioning, and the production of other cytokines by activated macrophages that further stimulate the activation of associated cells, including other macrophages, but also B and T lymphocyte populations

where they are responsible for their growth and differentiation (Secombes, 1991). Cytokines (including interleukins, interferons, TNF and MAF) are commonly targeted by components of tick salivary gland extracts that inhibit their functions (Wikel, *et al.*, 1996). In most instances the method of cytokine suppression is understood, such as the competitive occupation of receptor sites on host cells, but the exact biochemical identity of the active components is not known. Inhibition of cytokine functions effects the operation of specific immunity of hosts, preventing the establishment of an effective antibody response against the parasite, and also the hosts' cellular immunity, including macrophage chemotaxic and phagocytic abilities (Wikel, *et al.*, 1996). The observed effects of LIF on macrophage functions in this present study may, therefore, be caused not by the currently identified proteins, but by as yet unidentified molecules. Given the lack of progress in the identification of these molecules by the extensive research effort into tick immuno-modulators, these compounds in *L. salmonis*, if they do indeed exist, may not be classified for a significant length of time. However, the exhaustive knowledge base from the study of tick immunomodulation will benefit, and undoubtedly influence future studies of LIF's form, and functions within salmonids.

The discussions of the previous chapters have presented and explored hypotheses and speculations on; the mechanisms of copepodid host identification *in vitro*; the interactions, at the cellular level, within ASSE; the nature of LIF; and the physiological effects that LIF has on macrophages and their functions. In a preliminary and innovative study such as this, one that is designed around speculative concepts, the generation of significantly more questions than answers is only to be expected. However, the practical work completed within the project, in particular the development of ASSE, and the suggestion of, possibly wildly outrageous, hypotheses about unknown developmental and immunological mechanisms, and the presence of unidentified biologically active compounds, provides an "inspirational starting point" for future investigation, and the increased potential for further successes (*pers. comm.* Prof. C. Kennedy, Exeter University).

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APPENDICES

GLOSSARY OF TISSUE CULTURE TERMINOLOGY

Appendix 1

GLOSSARY OF TISSUE CULTURE TERMINOLOGY

Tissue culture is a specialist branch of the biological sciences which has become central to many fields of research and as such has acquired much varied terminology. However, the work of the Scottish scientist R. Ian Freshney, author of "Culture of Animal Cells: A Manual of Basic Technique" forms the basic reference source for most cell culturists and his terminology has been adopted throughout this thesis.

Cell culture	Growth of cells dissociated from the parent tissue by spontaneous migration or by mechanical or enzymatic dispersal.
Cell line	A propagated culture after the first subculture
Confluent	Where all the cells are in contact all around their periphery with other cells, and no available substrate is left uncovered
Continuous cell line	One having the capacity for infinite survival. Also known as 'established' and 'immortal'
Dedifferentiation	A term implying irreversible loss of the specialised properties that a cell would have expressed in vivo and the progressive loss of differentiated morphology
Epithelial	Used to describe any cultured cells of a polygonal shape with clear, sharp boundaries between cells. Also, describes cells derived from the epithelium.
Explant	A fragment of tissue transplanted from its original site and maintained in an artificial medium
Fibroblast	A proliferating precursor cell of the mature differentiated fibrocyte, but also used to describe cultured cells resembling fibroblasts – spindle shaped (bipolar) or stellate (multipolar), usually arranged in parallel arrays at confluence and possessing processes that exceed the nuclear

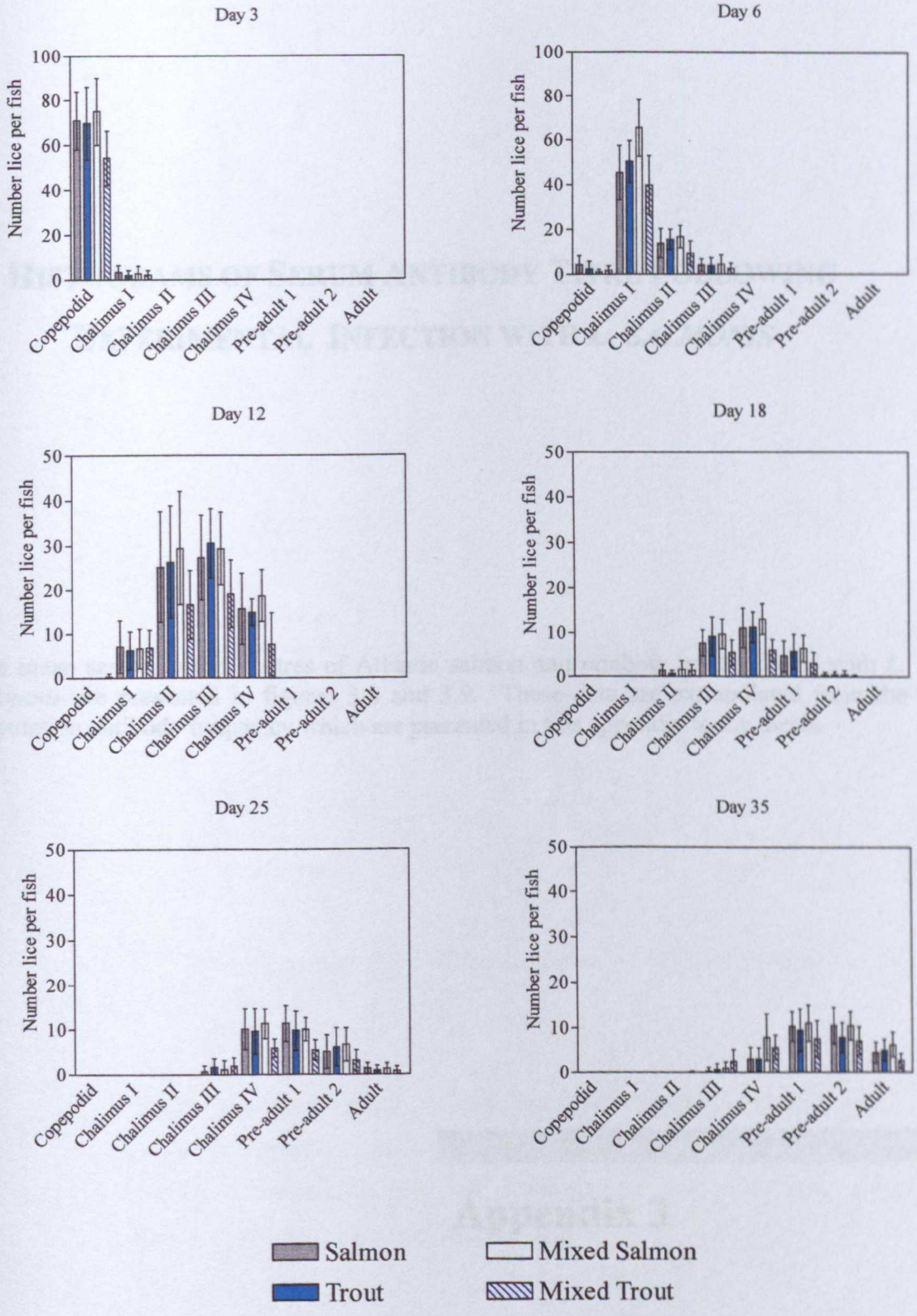
	diameter by at least three times
Finite cell line	A culture that has been propagated by subculture but is only capable of a limited number of cell generations *in vitro* before dying out
Histotypic	A culture resembling tissue-like morphology. A three dimensional culture recreated from dispersed cell culture that attempts to retain the tissue-like structure by cell proliferation and multi-layering
Medium	A mixture of organic salts and other nutrients capable of sustaining cell survival or proliferation *in vitro*
Organotypic	Histotypic culture involving the use of more then one cell type
Primary culture	A culture started from cells, tissues or organs taken directly from an organism – any culture before the first subculture
Passage	The transfer or subculture of cells from the culture vessel to another usually involving subdivision of a proliferating cell line
Tissue culture	The maintenance of tissue fragments *in vitro* but also generic term to include tissue explant culture, organ culture and dispersed cell culture

DEVELOPMENT OF *L. SALMONIS* ON ATLANTIC SALMON AND RAINBOW TROUT FOLLOWING EXPERIMENTAL INFECTION

These data are summarised in Chapter 3 in figures 3.1 and 3.2. They represent the development of *L. salmonis* on both salmonid species, experimentally infected with approximately 80 copepodid larvae, which were monitored at the time intervals indicated. The data are from fish maintained in single species tanks and in co-culture with the second species.

Appendix 2

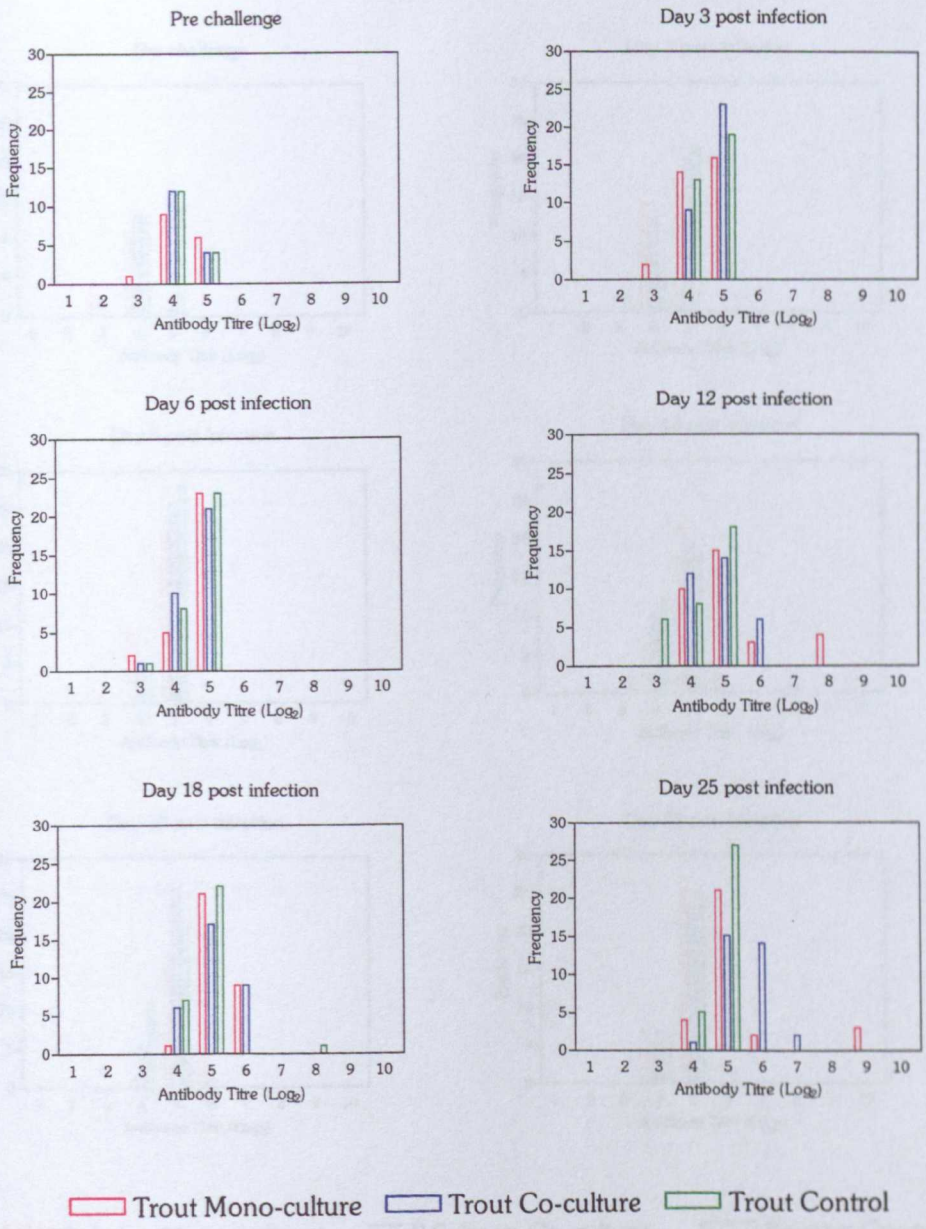
DEVELOPMENT OF L. SALMONIS ON ATLANTIC SALMON AND RAINBOW TROUT FOLLOWING EXPERIMENTAL INFECTION.



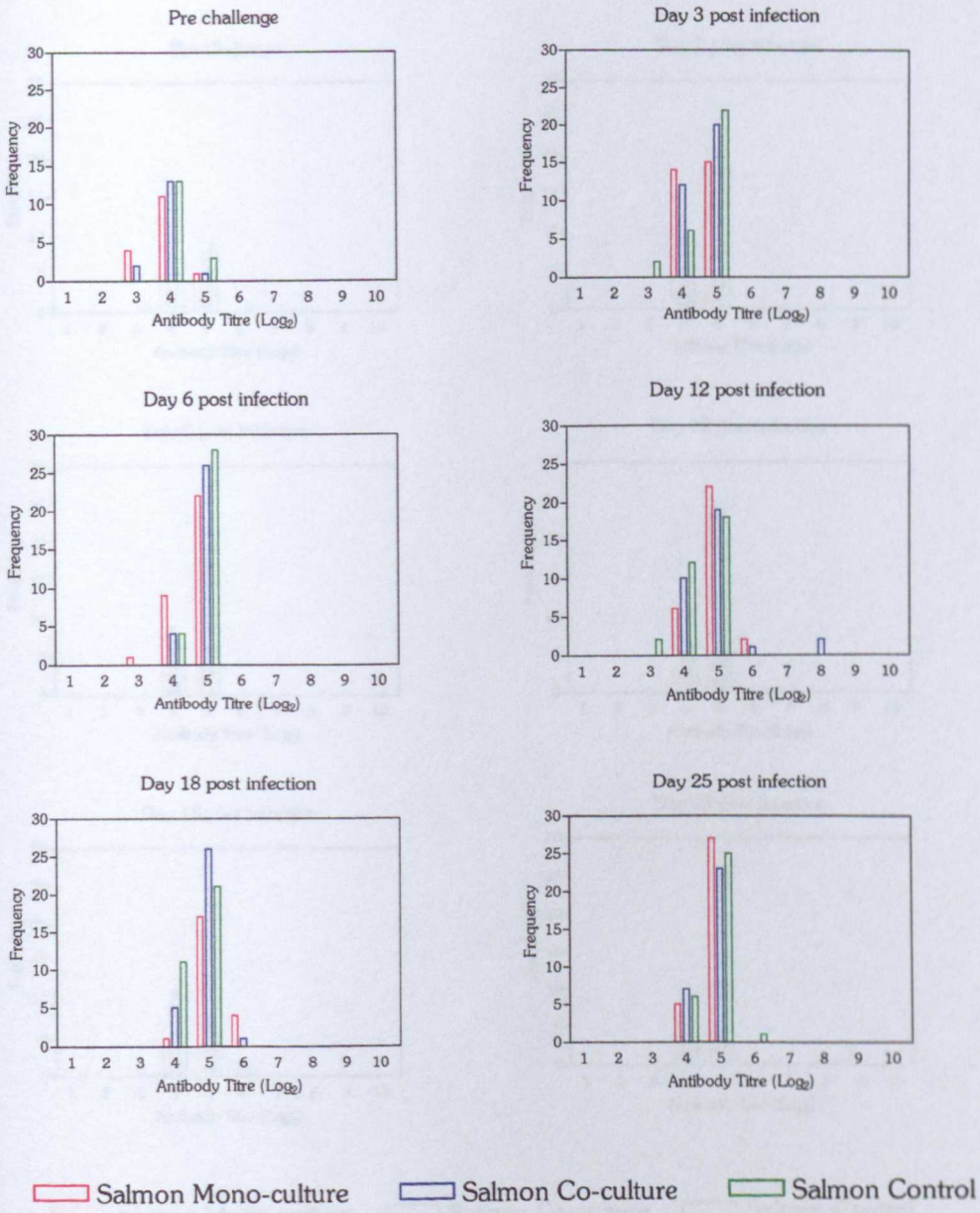
HISTOGRAMS OF SERUM ANTIBODY TITRE FOLLOWING EXPERIMENTAL INFECTION WITH *L. SALMONIS*

The mean serum antibody titres of Atlantic salmon and rainbow trout infected with *L. salmonis* are presented in figures 3.8 and 3.9. These data are extrapolated from the population antibody frequency which are presented in this appendix to the thesis.

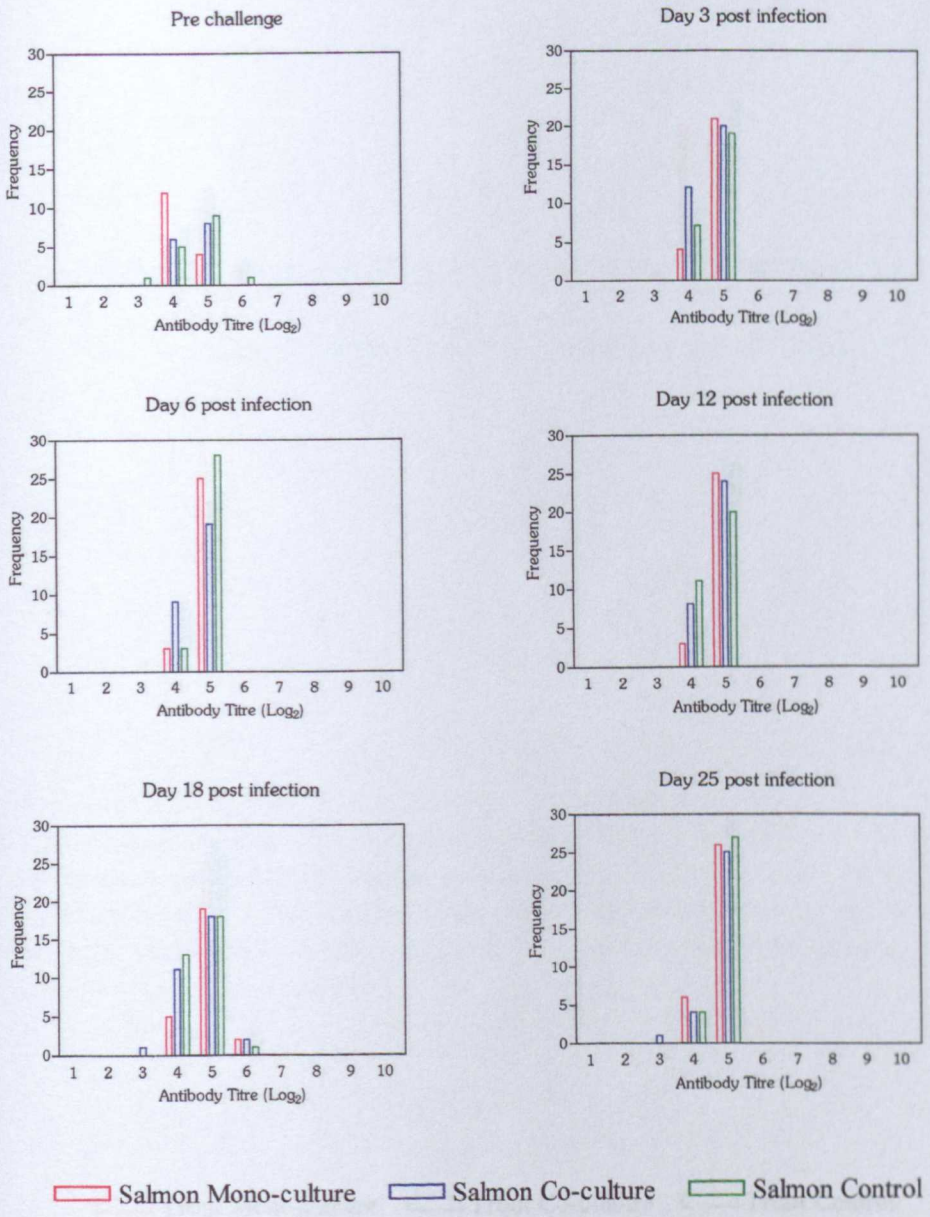
Appendix 3



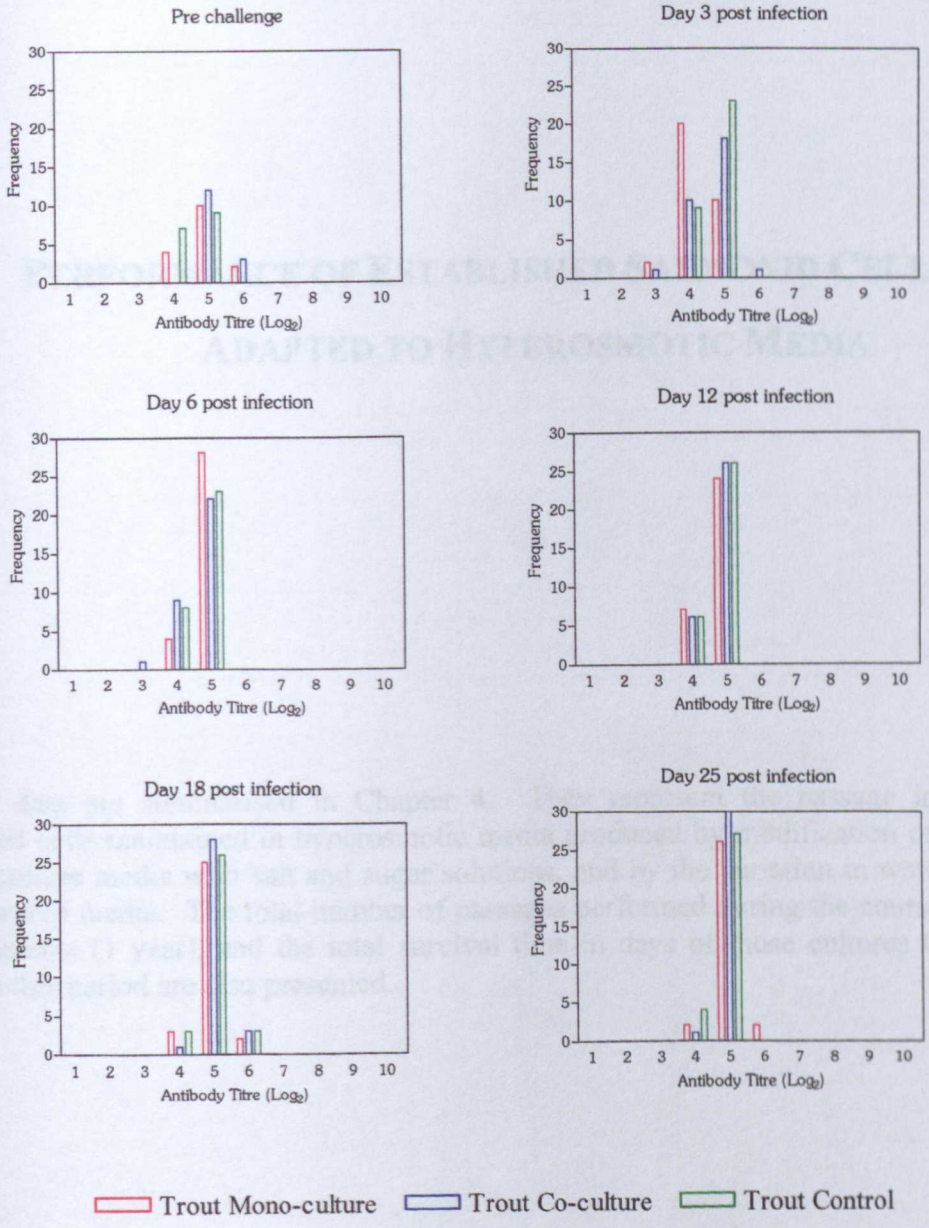
Frequency of agglutinating antibody titre (Log_2) against louse homogenate coated latex beads in trout serum following experimental infection with *L. salmonis*. Pre-challenge n=8 (with duplicates) for each group, n=16 (with duplicates) for each group from days 3 to 25.



Frequency of agglutinating antibody titre (Log_2) against louse homogenate coated latex beads in salmon serum following experimental infection with *L. salmonis*. Pre-challenge n=8 (with duplicates) for each group, n=16 (with duplicates) for each group from days 3 to 25.



Frequency of agglutinating antibody titre (Log_2) against *Aeromonas salmonicida* in salmon serum following experimental infection with *L. salmonis*. Pre-challenge n=8 (with duplicates) for each group, n=16 (with duplicates) for each group from days 3 to 25.



Frequency of agglutinating antibody titre (Log_2) against *Aeromonas salmonicida* in trout serum following experimental infection with *L. salmonis*. Pre-challenge n=8 (with duplicates) for each group, n=16 (with duplicates) for each group from days 3 to 25.

PERFORMANCE OF ESTABLISHED SALMONID CELL LINES ADAPTED TO HYPEROSMOTIC MEDIA

These data are summarised in Chapter 4. They represent the passage interval of cultured cells maintained in hyperosmotic media produced by modification of standard L-15 culture media with salt and sugar solutions, and by the variation in water content of powered media. The total number of passages performed during the course of these experiments (1 year), and the total survival time in days of those cultures that failed within this period are also presented.

Appendix 4

GROWTH AND PASSAGE CHARACTERISTICS OF CULTURED CELLS ADAPTED TO HYPEROSMOTIC MEDIA

ESTABLISHED SALMONID CELL LINES								
	ASE-W		RTG-2		CHSE		AS-6	
Media	Passage Interval (days)	Number Passages (1 year)*	Passage Interval (days)	Number Passages (1 year)	Passage Interval (days)	Number Passages (1 year)	Passage Interval (days)	Number Passages (1 year)
Salt Modified								
Normal	10.51	34.7	10.33	35.3	10.32	35.3	10.62	34.3
500	12.12	30.1	12.00	30.4	11.98	30.4	12.38	29.4
600	20.41	17.8	19.87	18.3	20.18	18	19.71	18.5
700	32.00	11[a] (352)	31.66	10[a] (316)	32.00	10[a] (320)	33.12	8[a] (265)
800	0	0	0	0	0	0	0	0
Sugar Modified								
Normal	10.62	34.3	10.27	35.5	10.31	35.4	10.47	34.8
500	12.93	28.2	11.88	30.7	13.40	27.2	12.95	28.1
600	21.61	16.8	20.84	17.5	18.99	19.2	21.55	16.9
700	34.10	10.7	30.88	10[a] (308)	36.51	9[a] (328)	33.33	9[a] (299)
800	0	0	0	0	0	0	0	0
Salt:Sugar Modified								
Normal	10.40	35.0	11.00	33.1	10.60	34.4	10.15	35.9
500	13.30	27.4	12.22	29.8	11.51	31.7	12.50	29.2
600	22.41	16.2	21.11	17.2	20.63	17.6	20.47	17.8
700	33.62	10.8	31.51	11.5	31.80	10[a] (318)	33.11	11.0
800	0	0	0	0	0	0	0	0
Modified Powdered Media								
Normal	10.38	35.1	10.18	35.8	10.41	35.0	10.29	35.4
500	11.36	32.1	12.41	29.4	11.98	30.4	12.05	30.2
600	19.88	18.3	22.60	16.1	22.50	16.2	21.06	17.3
700	34.20	10.6	32.05	8[a] (256)	31.55	11.5	31.22	9[a] (280)
800	0	0	0	0	0	0	0	0

* 1 year is 365 days

[a] Cell lines not viable after this passage. Figure in parentheses are the total number of days the adapted cells survived

Salt modified media are prepared by the addition of a solution of 50% v/v Magnesium chloride and 50% v/v Sodium sulphate to standard L-15 culture media

Sugar modified media are prepared by the addition of 1M sorbitol, 1M mannitol and 1M xylose each at 33.3% v/v to standard L-15 culture media

Salt-sugar modified media are prepared by the addition of 50% v/v salt solution and 50% v/v sugar solution to standard L-15 culture media

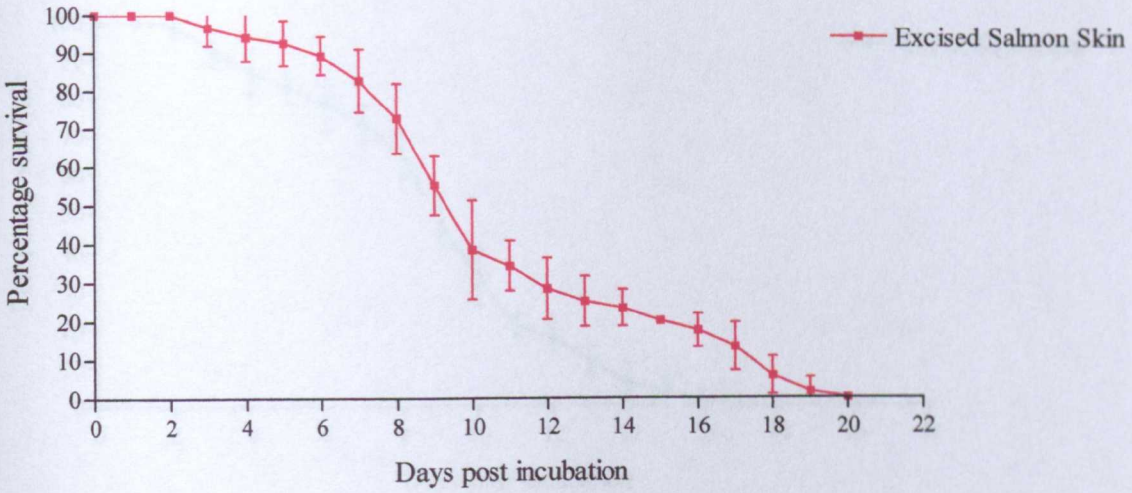
Modified powered media is prepared by the variation of the volume of water added to powered L-15 culture media (Sigma)

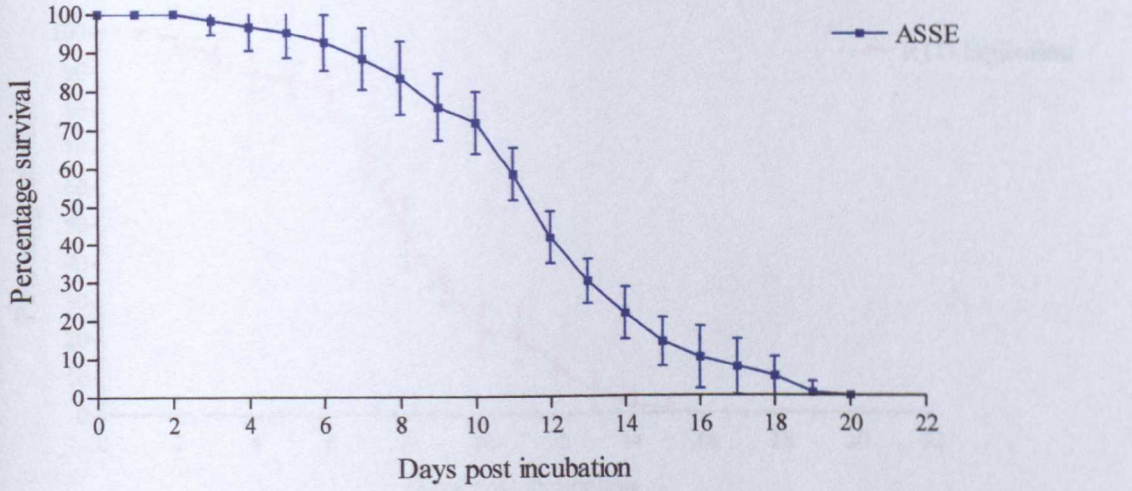
DATA OF COPEPODID SURVIVAL DURING *IN VITRO* CULTURE CYCLES

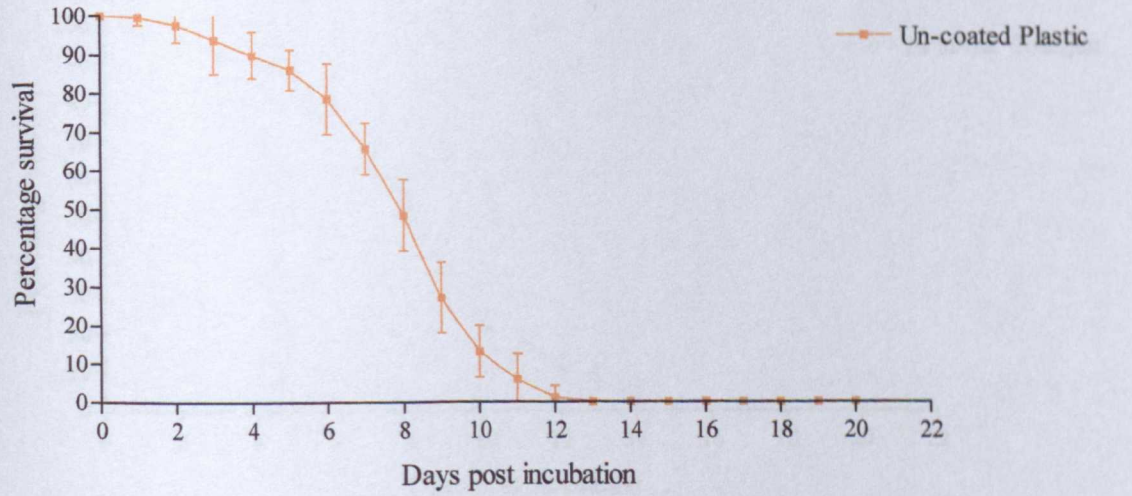
These data are summarised in Chapter 5. In the body of the thesis they are presented without error bars to allow better comparison of the results of louse survival on each culture substrate. In this appendix the data are presented graphically and are the mean of 96 observations at each time point ± standard deviation in experiments with standard substrates, and the mean of 12 observations at each time point ± standard deviation in experiments using substrates supplemented with mucus, peptone and methionine. In all cases, each observation recorded the survival of sub-populations of 10 copepodids.

Appendix 5

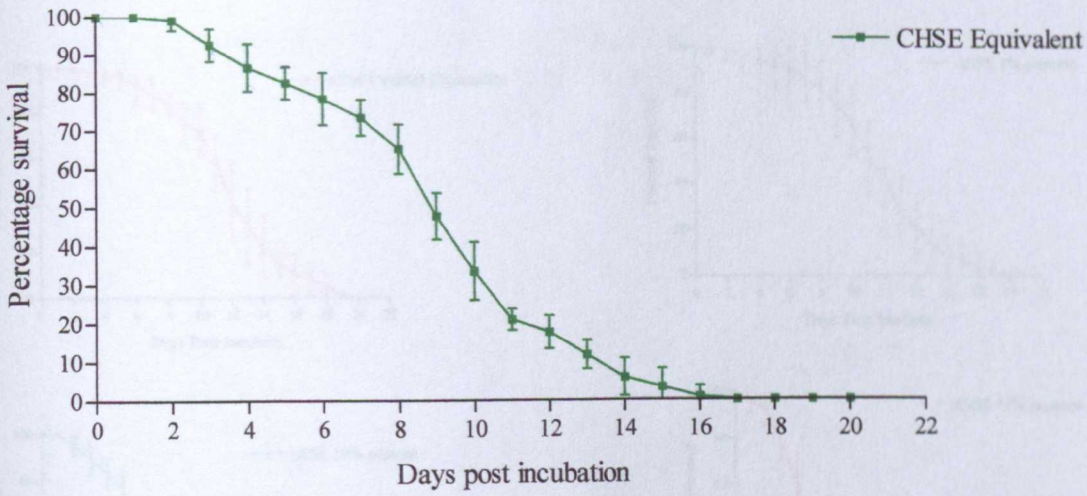
COPEPODID SURVIVAL DURING STANDARD CULTURE CYCLE

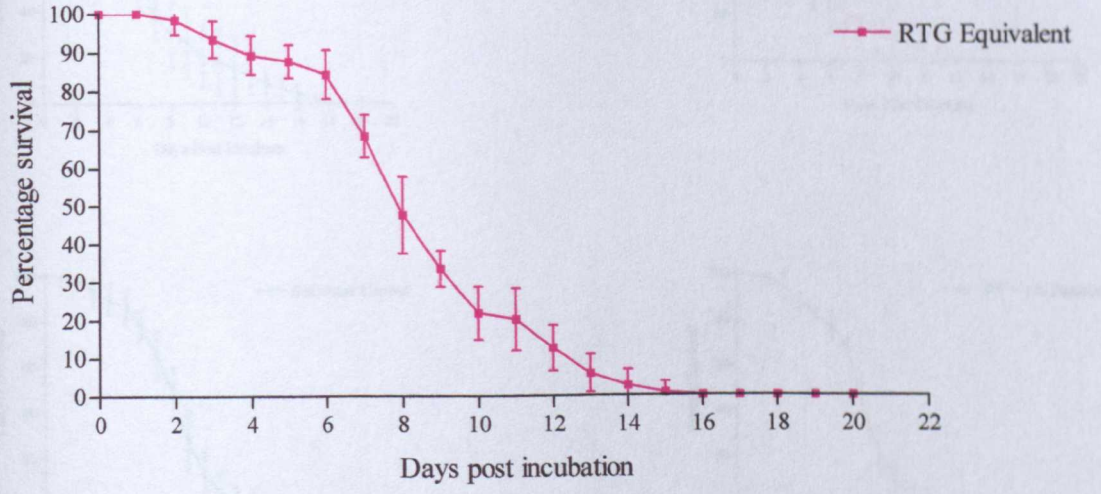


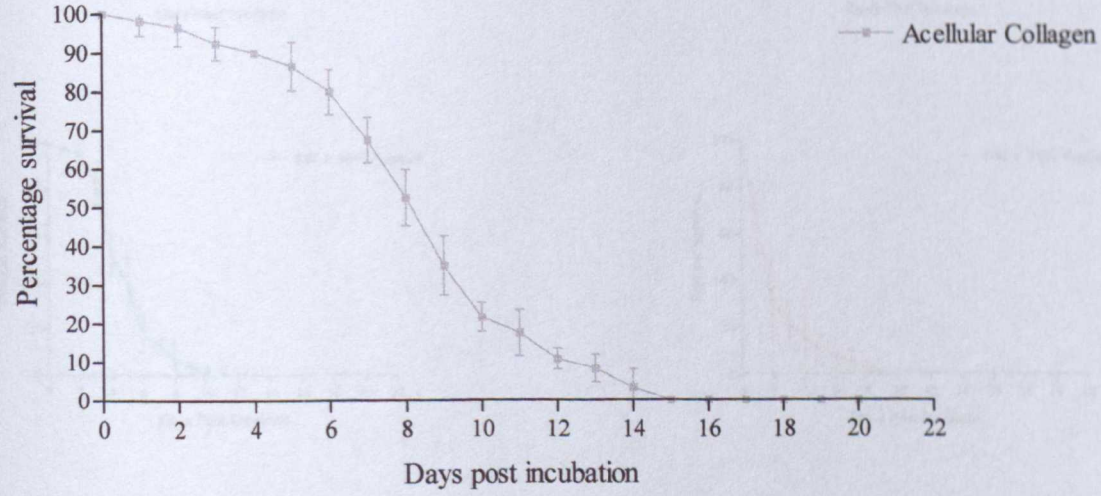




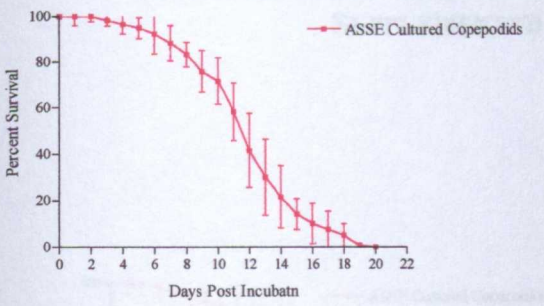
COPEPODID SURVIVAL DURING STANDARD CULTURE CYCLE

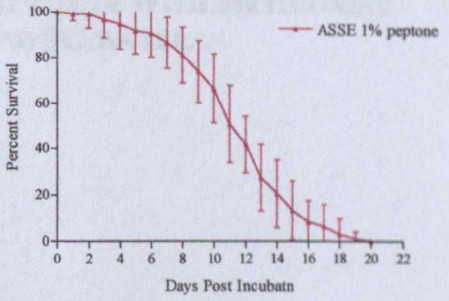


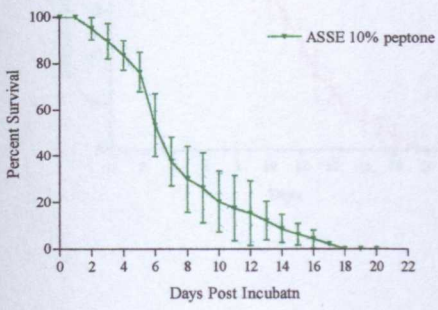


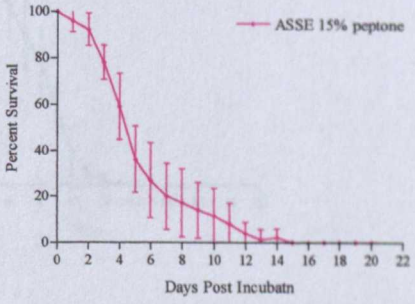


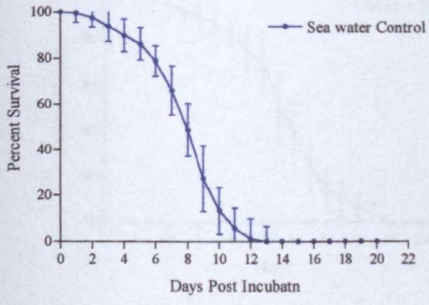
COPEPODID SURVIVAL DURING CULTURE CYCLE WITH PEPTONE SUPPLEMENTED ASSE AND SEAWATER

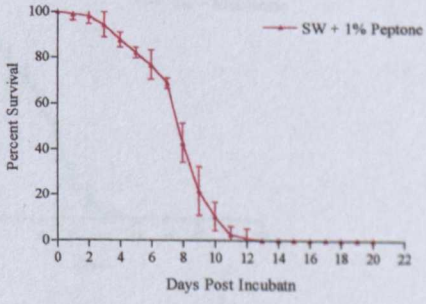


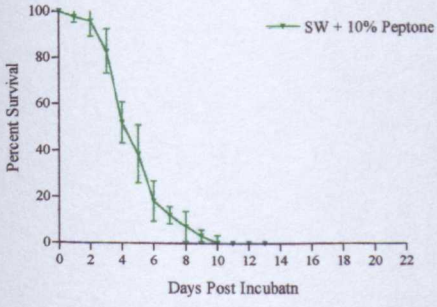


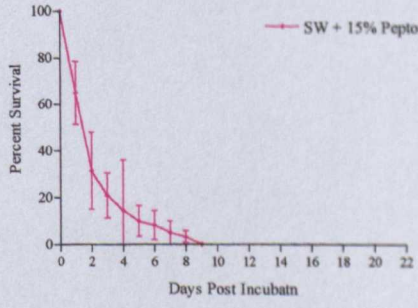




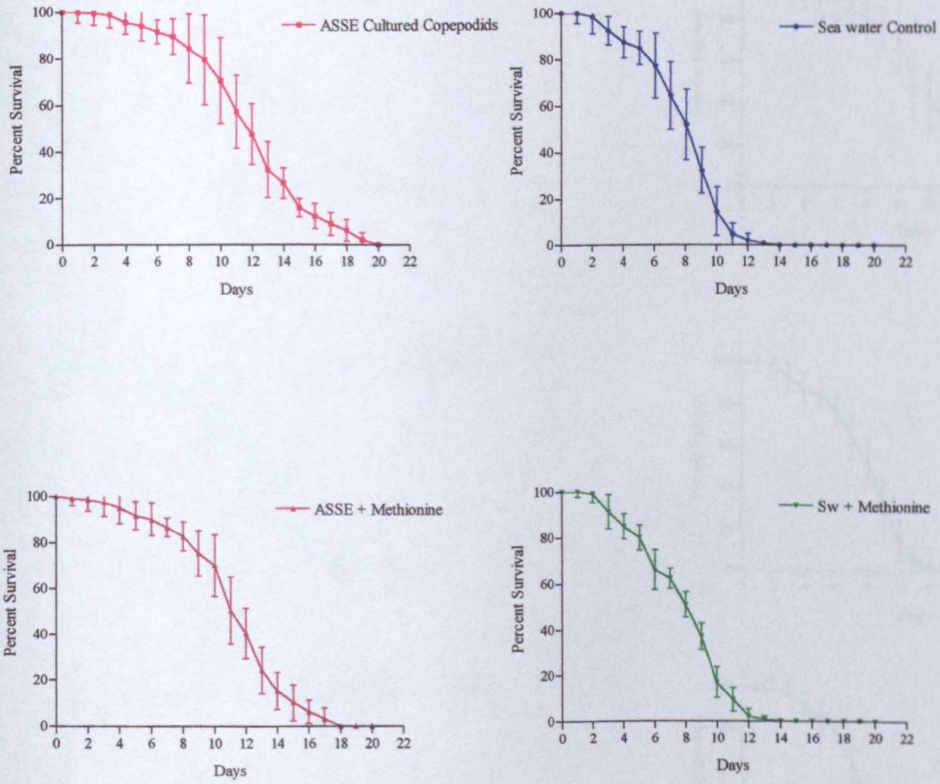








COPEPODID SURVIVAL DURING CULTURE CYCLE WITH METHIONINE SUPPLEMENTED ASSE AND SEAWATER



COPEPODID SURVIVAL DURING CULTURE CYCLE WITH MUCUS SUPPLEMENTED ASSE AND SEAWATER